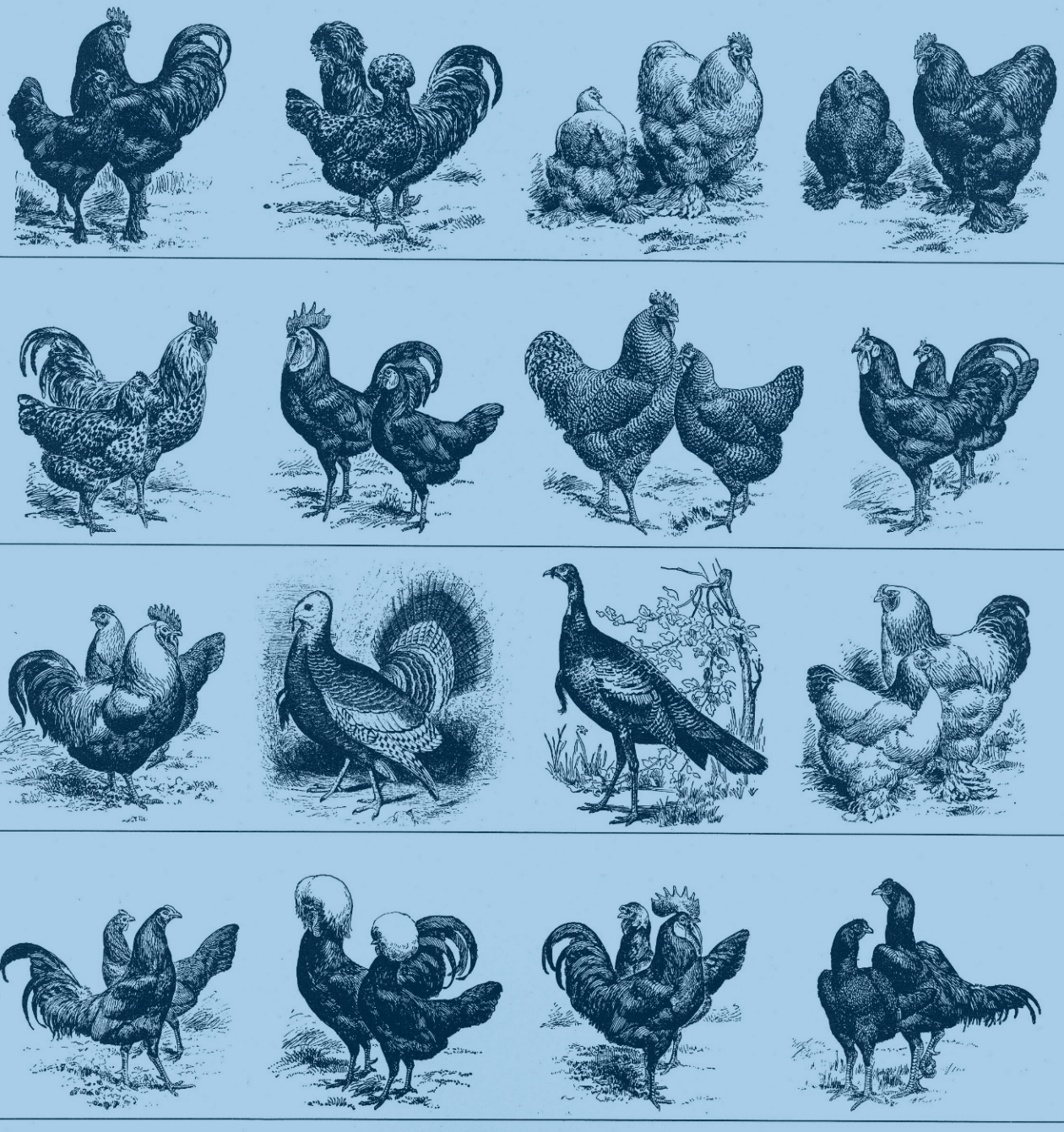
PROCEEDINGS OF THE SEVENTY-FIRST WESTERN POULTRY DISEASE CONFERENCE

April 3-6, 2022 Vancouver, BC, Canada

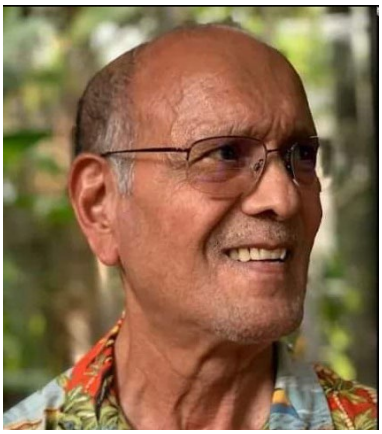
WESTERN POULTRY
DISEASE CONFERENCE



PROCEEDINGS OF THE SEVENTY-FIRST WESTERN POULTRY DISEASE CONFERENCE

April 3-6, 2022 Vancouver, BC, Canada

IN MEMORIAM
A. SINGH DHILLON



Dr. Avtar Singh Dhillon passed away March 19, 2022 in Portland, Oregon. He was born and raised in India and obtained his DVM degree from Punjab University. After his graduation, he was selected from a pool of 2000 veterinarians in the Indian army as a commissioned officer and served in the army for six years.

Dr. Dhillon moved to the USA to advance his education. In 1972, he completed a MS, and in 1975, he graduated with a PhD degree. Both degrees are from the University of Wisconsin in Madison. After his PhD graduation, Singh worked for five years at the Animal Disease Diagnostic Laboratory, West Lafayette, Indiana as an avian pathologist. In 1981, he and his family moved to the state of Washington. He served as a professor for Washington State University and as lab director of the Avian Health and Food Safety Laboratory (WADDL) for 28 years.

Dr. Dhillon was a mainstay of poultry veterinary medicine and diagnostics in the Pacific Northwest for many years. He was the recipient of the certificate of excellence in a scientific research paper at Poultry Science Association Annual Meeting in 1974. In 2009 Singh was honored as the Pacific Egg & Poultry Association's Scientist of the Year. He served as Program Chair and President of the Western Poultry Disease Conference in 1993-94, and in 2013 received the Special Recognition Award at the 62nd WPDC.

Many who have known Singh on a more personal level attest to a kind and caring person that went far beyond simple accomplishments in veterinary medicine. First and foremost, he was a dedicated family man. His son comments that "my father might've come to this land with $50 in his pocket, but through shear grit, he made a treasured, financially stable, and spiritually rich life – not just for himself, or his family, or his extended family, but for almost everyone he talked to."

In the many years that he lived in Puyallup, he participated in multiple public boards, including the Senior Center Advisory Board for the Puyallup School System He was active in the Eye Opener Toastmaster Club, and served as president of the organization in 1988.

For those young students and poultry veterinarians who had the privilege of knowing Dr. Dhillon, he was a great mentor and had lots of fun stories to tell. Dr. Rocio Crespo, who worked with Singh in the Puyallup lab, recounts the following:

> I first met him when I was doing my residency in Fresno. Dr. Dhillon was one of those professors who always asked the tough questions, so I was scared of him. Over time I came to know him better and realized what a generous and kind person he was. He loved his work and the people that worked with him. When I started working in Puyallup, he introduced me to the producers in the area and showed some of the tricks to be successful in the lab. During the first year, he would come every day into the lab and we would chat about cases, people, finances, research, etc. Over time his visits became less frequent, but he always asked me to give him a tour of the lab because I kept changing rooms and equipment. Even though he was retired, when I was on vacation, he would cover the cases for me and would not ask for any compensation. Besides being a great microbiologist and diagnostician, he made his own wine (boy that was good!). He also cooked excellent chicken.

Dr. Dhillon will be greatly missed by many friends, colleagues, the poultry industry, and veterinary poultry diagnostic medicine in general.

71st WPDC CONTRIBUTORS LIST

(as of March 24, 2022)

We wish to give a heart-felt thanks to these sponsors listed on the next few pages that have contributed so generously and given their 2022 pledge to the 71st Western Poultry Disease Conference, and to those who give their continual support over the years!

SUPER SPONSORS

BENFACTORS

Hygieia Labs

Adventures in Biotechnology

PATRONS

ONE FAMILY.
ONE PURPOSE.

Boehringer/Ingelheim

Evonik Corporation

Laboratorio Avi-Mex SA de CV
Ciudad de México, CDMX, México

DONORS

Hidden Villa Ranch/Nest Fresh
Fullerton, CA

Vega Farms
Dixon, CA

Veterinary Diagnostic Pathology, LLC
Fort Valley, VA

SUSTAINING MEMBERS

Canadian Poultry Consultants, Ltd
Abbotsford, BC, Canada

J.S. West Milling Co.
Modesto, CA

Rodrigo Gallardo
Poultry Medicine Laboratory at UCD

SPECIAL ACKNOWLEDGEMENTS

The 71st Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and help defray some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international meeting. Many companies and organizations have once again given substantial financial support, including some that also send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

Once again, the WPDC is forever grateful to our distinguished contributors and supporters of the conference who are vital in making the conference a success. Our contributors and supporters are listed within the pages of these proceedings. We greatly appreciate their generosity and sincerely thank them and their representatives for supporting this year's meeting.

We acknowledge all of our WPDC colleagues, especially our attendees that can join us for our first in-person meeting since 2019. We remain hopeful that we can continue the in-person meeting format in future years, and we thank everyone who has accommodated the changes we've implemented in order to promote a safe and productive meeting.

The Executive Committee has worked tirelessly this year in order to achieve 501(c)(3) nonprofit status for the WPDC. We especially acknowledge the efforts of Nancy Reimers and Rodrigo Gallardo in making this longtime goal a reality. This WPDC Foundation will allow us to administer more efficiently and give more opportunities to students and members.

Many have provided special services that contribute to the continued success of this conference. For this year's meeting, the WPDC has contracted BK Association Management, Jacksonville, Florida for providing registration and logistical support. We especially thank Ms. Channah Pool, for her helpful assistance. We acknowledge the AAAP as well as the WPDC Executive Committee and Board of Directors for their support in organizing this meeting. Again, we thank Bob and Janece Bevans-Kerr for their continual support, and wish them well in their retirement.

We thank Dr. David Frame for editing and producing another outstanding Proceedings of this meeting. Dr. Frame is indebted to Mr. Dana Frame for his meticulous proofreading and formatting the Proceedings for publication. We express our gratitude to all authors who submitted manuscripts, and are especially appreciative of those who submitted their manuscripts on time. Once again, we acknowledge Bruce Patrick (Graphic Communications, Brigham Young University) for the front page cover design displayed in the electronic proceedings.

WESTERN POULTRY
DISEASE CONFERENCE

We wish to express a very special thanks to the ACPV for managing our proceedings on the web site and facilitating RACE-approved CE credit for attendance at the WPDC!

71st WESTERN POULTRY DISEASE CONFERENCE OFFICERS

PRESIDENT
Simone T. Stoute
California Animal Health and Food Safety
Turlock Branch, Turlock, CA 95381
Phone: (209) 634 5837
ststoute@ucdavis.edu

PROGRAM CHAIR
Simone T. Stoute
California Animal Health and Food Safety
Turlock Branch, Turlock, CA 95381
Phone: (209) 634 5837
ststoute@ucdavis.edu

PROGRAM CHAIR-ELECT
Carmen Jerry
UC Davis, CAHFS Turlock Laboratory
cfjerry@ucdavis.edu
Phone: (209) 634- 5837

EXECUTIVE SECRETARY
Shelly Popowich
Western College of Veterinary Medicine
University of Saskatchewan
shelly.popowich@usask.ca

CONTRIBUTIONS CHAIR
Nancy Reimers
Cutler Associates International
nancyreimers@gmail.com

CONTRIBUTIONS SUPPORT
Rich Chin
rpchin@ucdavis.edu

PROCEEDINGS EDITOR
David D. Frame
Utah State University
Central Utah Veterinary
Diagnostic Laboratory
514 West 3000 North
Spanish Fork, UT 84660
david.frame@usu.edu

SECRETARY-TREASURER
Rodrigo A. Gallardo
University of California, Davis
4009 VM3B
Davis, CA95616
Phone: (530) 219 4963
ragallardo@ucdavis.edu

WPDC FOUNDATION BOARD OF DIRECTORS

Ana Paula Da Silva
David D. Frame
Rodrigo A. Gallardo
Shelly Popowich
Nancy Reimers
Simone Stoute

71st WPDC PROCEEDINGS

Please note that the proceedings of the 71st Western Poultry Disease Conference are not refereed, but are presented as a service and a source of information to those attending the conference and to others who wish to gain some insight as to the information presented.

The proceedings of the 71st WPDC are available in electronic format only. They can be downloaded from the American College of Poultry Veterinarians website (www.acpv.info).

WESTERN POULTRY DISEASE CONFERENCE (WPDC) HISTORY

YEAR	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
1st WPDC – 1952		A. S. Rosenwald		
2nd WPDC – 1953	P. D. DeLay	A. S. Rosenwald		
3rd WPDC – 1954	C. M. Hamilton	Kermit Schaaf		
4th WPDC – 1955	E. M. Dickinson	W. H. Armstrong		
5th WPDC – 1956	D. E. Stover	E. E. Jones		
6th WPDC – 1957	D. V. Zander	H. E. Adler		
7th WPDC – 1958	H. E. Adler	E. E. Jones		
8th WPDC – 1959	R. D. Conrad	L. G. Raggi		
9th WPDC – 1960	L. G. Raggi	A. S. Rosenwald		
10th WPDC – 1961	A. S. Rosenwald	D. V. Zander		
11th WPDC – 1962	D. V. Zander	R. V. Lewis		
12th WPDC – 1963	R. V. Lewis	Walter H. Hughes		
13th WPDC – 1964	W. H. Hughes	Bryan Mayeda		
14th WPDC – 1965	B. Mayeda	R. Yamamoto		
15th WPDC – 1966	R. Yamamoto	David S. Clark (1st sign of Contributors)		
16th WPDC – 1967	D. S. Clark	Roscoe Balch		
17th WPDC – 1968	R. Balch	Richard McCapes		
18th WPDC – 1969	R. McCapes	Dean C. Young		
19th WPDC – 1970 4th Poultry Health Sym. (PHS)	D. C. Young	W. J. Mathey	1st combined WPDC & PHS	1st listing of distinguished members
20th WPDC – 1971 5th PHS	W. J. Mathey	Ramsay Burdett		
21st WPDC – 1972 6th PHS	R. Burdett	Marion Hammarlund		
22nd WPDC – 1973 7th PHS	M. Hammarlund	G. W. Peterson		
23rd WPDC – 1974 8th PHS	G. W. Peterson	Craig Riddell		
24th WPDC – 1975 9th PHS	C. Riddell	Ralph Cooper		
25th WPDC – 1976 10th PHS	R. Cooper	Gabriel Galvan		
26th WPDC – 1977 11th PHS	G. Galvan	Don H. Helfer	Hector Bravo	
27th WPDC – 1978 12 PHS	D. H. Helfer	Art Bickford		
28th WPDC – 1979 13th PHS	A. Bickford	J. W. Dunsing		
29th WPDC – 1980 14th PHS 5th ANECA	J. W. Dunsing Angel Mosqueda T.	G. Yan Ghazikhanian	P. P. Levine	
30th WPDC – 1981 15th PHS	G. Y. Ghazikhanian	Mahesh Kumar		
31st WPDC – 1982 16th PHS	M. Kumar	Robert Schock		
32nd WPDC – 1983	R. Schock	George B. E. West		
33rd WPDC – 1984	G. B. E. West	Gregg J. Cutler		
34th WPDC – 1985	G. J. Cutler	Don W. Waldrip		Bryan Mayeda

YEAR	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
35th WPDC – 1986	D. W. Waldrip	Duncan A. McMartin	J. A. Allen	
11th ANECA	Jorge Basurto	Mario Padron	A. Tellez-G. Rode	
36th WPDC – 1987	D. A. McMartin	Marcus M. Jensen		
37th WPDC – 1988	M. M. Jensen	Barry Kelly	A. S. Rosenwald	
38th WPDC – 1989	B. Kelly	Masakazu Matsumoto		Louise Williams
39th WPDC – 1990	M. Matsumoto	Jeanne M. Smith		Dean Young
40th WPDC – 1991	J. M. Smith	Richard P. Chin	A. S. Rosenwald	
16th ANECA	Martha Silva M.	David Sarfati M.	A. S. Rosenwald	
41st WPDC – 1992	R. P. Chin	Rocky J. Terry	Marcus Jensen	Henry E. Adler * *(posthumous) R. A. Bankowski C. E. Whiteman
42nd WPDC – 1993	R. J. Terry	A. S. Dhillon	W. W. Sadler	Royal A. Bagley
43rd WPDC – 1994	A. S. Dhillon	Hugo A. Medina		G. B. E. West
44th WPDC – 1995	H. A. Medina	David D. Frame	W. M. Dungan* *(posthumous)	A. J. DaMassa Gabriel Galvan Walter F. Hughes W. D. Woodward R. Yamamoto
45th WPDC – 1996	D. D. Frame	Mark Bland	Don Zander	Pedro Villegas
21st ANECA	R. Salado C.	G. Tellez I.	M. A. Marquez	Ben Lucio M. Mariano Salem Victor Mireles Craig Riddell
46th WPDC – 1997	Mark Bland	James Andreasen, Jr.	Bryan Mayeda	Roscoe Balch Paul DeLay J. W. Dunsing Don Helfer D. E. Stover
47th WPDC – 1998	J. Andreasen, Jr.	H. L. Shivaprasad	W. J. Mathey	Marcus Jensen Duncan Martin
48th WPDC – 1999	H. L. Shivaprasad	R. Keith McMillan		
49th WPDC – 2000	R. K. McMillan	Patricia Wakenell	R. P. Chin	Ralph Cooper Robert Tarbell
50th WPDC – 2001	P. Wakenell	Ken Takeshita		Don Bell Art Bickford
51st WPDC – 2002	K. Takeshita	Barbara Daft	Hiram Lasher	Bachoco S.A. de C.V.
27 ANECA	J. Carillo V.	Ernesto P. Soto		Productos Toledano S.A.
52nd WPDC – 2003	B. Daft	David H. Willoughby		Roland C. Hartman
53rd WPDC – 2004	D. H. Willoughby	Joan Schrader		G. Yan Ghazikhanian
54th WPDC – 2005	J. Schrader	Stewart J. Ritchie	W.D. Woodward	R. Keith McMillan
55th WPDC – 2006	S. J. Ritchie	Peter R. Woolcock		M. Hammarlund
56th WPDC – 2007	P.R. Woolcock	Bruce Charlton	R. Keith McMillan	M. Matsumoto
57th WPDC – 2008	B. Charlton	Rocio Crespo	A. S. Rosenwald* *(posthumous)	B. Daft
33rd ANECA	M. A. Rebollo F.	Maritza Tamayo S.	A. S. Rosenwald*	Ernesto Ávila G.
58th WPDC – 2009	R. Crespo	Victoria Bowes		G.L. Cooper
59th WPDC - 2010	V. Bowes	Nancy Reimers		
60th WPDC - 2011	N. Reimers	Larry Allen		John Robinson
61st WPDC - 2012	L. Allen	Vern Christensen		
62nd WPDC - 2013	V. Christensen	Portia Cortes	Víctor Manuel Mireles M.	A. Singh Dhillon

YEAR	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
63rd WPDC – 2014	P. Cortez	Ernesto Soto	Hugo Medina	
39th ANECA	Néstor Ledezma M.	Ernesto Soto	Benjamin Lucio Martínez	
64th WPDC – 2015	Ernesto Soto	Shahbaz Haq	Bruce R. Charlton	David Willoughby
65th WPDC – 2016	S. Haq	Susantha Gomis		
66th WPDC – 2017	S. Gomis	C. Gabriel Sentíes-Cué	Richard McCapes	Peter Woolcock Richard P. Chin
67th WPDC – 2018	C.G. Sentíes-Cué	Rodrigo A. Gallardo		David D. Frame
68th WPDC – 2019	R. Gallardo	Sarah Mize		Gregg J. Cutler
44th ANECA	Ricardo Cuetos Collado	Maritza Tamayo		
69th WPDC – 2020	S. Mize	Simone T. Stoute		Mark C. Bland
70th WPDC – 2021	S. Stoute	Lynn G. Bagley	Walter F. Hughes	H. L. Shivaprasad
71st WPDC – 2022	S. Stoute	Simone T. Stoute		

MINUTES OF THE 70TH WPDC ANNUAL BUSINESS MEETING

Secretary-Treasurer Gallardo called the meeting to order on Tuesday, March 30, 2021, at 9:00 AM. The meeting was held virtually because of the ongoing COVID-19 pandemic. Although all membership was invited, this ended up being an Executive Committee meeting (no general members signed in). There were seven people in attendance.

APPROVAL OF 69th WPDC BUSINESS MEETING MINUTES

Because of the COVID-19 pandemic and the subsequent cancellation of the 70th in-person WPDC, a general business meeting was not held at the 70th WPDC; therefore, no minutes of the 69th WPDC were presented for approval.

ANNOUNCEMENTS

The Executive Committee – particularly Dr. Nancy Reimers and Dr. Rodrigo Gallardo – are actively pursuing a path for 501(c)(3) status for the WPDC.

The 71st WPDC will be held in-person April 3-6, 2022 at the Marriott Pinnacle Downtown in Vancouver, BC, Canada.

REPORT OF THE SECRETARY-TREASURER

Dr. R. Gallardo presented the Secretary-Treasurer report. He announced that there are 144 full registrants and 31 students for 2021. The registration income amounts to $25,028. Sponsorship income through contributions was $39,950. We currently have $145,701in the PHR SVM Account. We spent a total of $385 on plaques this year.

REPORT OF THE PROGRAM CHAIR

Dr. Lynn Bagley presented the Program Chair report. He announced that there were 54 titles accepted, two withdrawals, and two rejected. Number of uploaded talks was 48, and he had four invited speakers. Dr. Bagley noted that the virtual posters ended up being no different virtually than the oral presentations. He suggested to distinguish student presentations on the initial submission form.

REPORT OF THE PROCEEDINGS EDITOR

Dr. David Frame presented the Proceedings Editor report. He noted that the job load of Proceedings Editor is no different between an in-person and virtual meeting. Next year it will be anticipated to hyperlink the sponsors' logos in order to directly access company sites if so desired.

NEW BUSINESS

The following officers were nominated for 2022-2023:

Program Chair: Simone Stoute
President: Lynn Bagley
Contributions Chair: Nancy Reimers
Contributions Support: Rich Chin
Proceedings Editor: David Frame
Secretary-Treasurer: Rodrigo Gallardo
Program Chair-elect: Carmen Jerry
Executive Secretary: Shelly Popowich

Nominations for all officers were closed and all nominees were approved unanimously.

Future meetings:

It was voted to hold the 72nd WPDC, in 2023, in Sacramento, CA, at the Holiday Inn Sacramento Arena. The location for 73rd WPDC (in 2024) was discussed. It was concluded to have it in Salt Lake City, Utah, with the meeting venue to be determined according to bid.

2022: Vancouver, April 3-6

2023: Sacramento, March 12-15
2024: Salt Lake City, mid-April

Dr. Gallardo adjourned the meeting at 11:00 AM.

THE ARNOLD S. ROSENWALD LECTURE

C. Stephen Roney
2022

WESTERN POULTRY DISEASE CONFERENCE

RETROSPECTIVE PERSPECTIVES OF A POULTRY VETERINARIAN

C. Stephen Roney

CSR Poultry Health Consulting Services, Midland City, AL 36350

It is an extreme honor to deliver the Arnold S Rosenwald lecture for the 71st Western Poultry Disease Conference. Rosy was the very heart of this meeting for so many years; especially for the 35 years for which I have been attending. He was a great poultry veterinarian, mentor, colleague and friend. His presence is still felt by all who knew him so well.

The primary factor that attracted me to poultry veterinary medicine is the extreme variability in the type of work one might do. Career types might include:

Federal	Vaccine/Pharmaceutical Technical Service
State	Feed Additive Technical Service
University	Poultry Production
PHS, CDC	Private Practice/ Consulting
Military	Breeder/Genetics

Economic considerations, along with a deep sense of adventure, have allowed me to personally experience almost all of these disciplines. I might add that a very understanding spouse and family are required to move around as much as I have.
My goal in this presentation is to describe how I have seen each of these areas change in the 35 years for which I have been a poultry veterinarian. Looking back, it seems like only yesterday, but when I consider things now versus then, I am somewhat bewildered. I am sure a lot of things are better and they certainly are different.

I primarily became a poultry veterinarian due to the NPIP. I was working with APHIS, testing cows for brucellosis and TB in Louisiana, when they decided they needed a veterinarian to take the position of NPIP senior coordinator upon the retirement of Dr. Irving Peterson. I applied and was accepted to the MAM program under contract with APHIS to move to Maryland and work in poultry.

Of course, the NPIP was formed in 1935 to control pullorum disease and is a state-federal-industry cooperative program unlike any other in the world. The program started with *Salmonella* Pullorum/Typhoid certification but later added mycoplasma for breeder bird certification. Later on, a *Salmonella* Enteritidis (SE) program was added for commercial layers followed by the AI program. SE and AI changed the NPIP from a breeder bird program to encompassing all levels of commercial poultry production. This has been quite a challenge for APHIS to incorporate. Biennial conferences produced changes in legislation creating the program standards which streamlined the NPIP's ability to adapt to the industry. Recently, compartmentalization of primary breeders has been added resulting in protection of our breeding stock in the event of a disease outbreak,

As Veterinary Coordinator and then Senior Coordinator, I was actively involved in an investigational audit by the office of General Council, the establishment of Program Standards, working towards online NPIP form availability with electronic registration, the SE program, the AI program and the introduction of compartmentalization for primary breeders.

State employment for poultry vets primarily involves working in poultry diagnostic labs. I have had the opportunity to be the director of two labs in Alabama and of the PDRC Diagnostic Lab at UGA. States seem to be forever looking for money to fund these labs. Many times, they get the money to build a nice facility but then cannot get the legislature to fund salaries for adequate employees to staff them. Diagnostic labs serve a vital function for the poultry industry and many are authorized labs

of the NPIP. The poultry industry should make a point to encourage state governments to fund staffing for these labs and make sure that the positions remain financially attractive to attract high quality poultry veterinarians.

I have had the opportunity to teach poultry science and poultry medicine at two different institutions some 30 years apart. Most recently teaching avian medicine at UGA and formerly teaching basic poultry science at Snead State College in Boaz, AL. While college students have remained the same in many ways, some differences have developed in how they learn and how we teach them. Most recently, Covid-19 has changed all areas of education by making virtual instruction a norm. On-line avian medicine programs now exist and those of us who learned our skills through organoleptic instruction wonder how effective these are and how this will affect the veterinary profession in the future. We now see less hands-on diagnostics and more metanomic analysis than in the past and this is apt to continue and increase in the future.

I started my poultry medicine career with an international allied company based in the US. In the 1980s, many allied companies were US owned with subsidiaries around the world. This meant that an employed veterinarian would spend some time with the US poultry industry but would also spend considerable time travelling internationally visiting the other offices. Technical service then in the US consisted of riding with live operations personnel, usually a serviceperson. I have spent numerous days visiting farms on a serviceperson's route and then having a summary meeting with the live operations manager and sometimes the complex manager and nutritionists afterwards. At that time, we would do the occasional posting session (broiler health survey) if needed. Now it seems that posting sessions have become a necessary and standardized method of analysis of poultry health in most broiler companies. Allied companies are not fond of providing veterinary personnel for these sessions but so far no one has been able to offer an accepted alternative. Poultry veterinary technical service continues to evolve in order to offer the most assistance to the live production veterinarian.

The live production veterinary position was once the most coveted spot for upcoming poultry vets. They were the ones who made the critical decisions and thus commanded respect. They were also the recipients of the many gifts and entertainment that were once part of the allied/production relationship. Today there is much less entertainment and other perks than in the past and the added pressure of some of the production positions makes these jobs less desirable for the young people entering the profession. Production veterinarians spend much less time in the chicken houses now and more time in meetings such as strategic planning and Agri Stats analysis. I saw Agri Stats begin and grow in this industry. It once became such an overwhelming influence that some of the input numbers started to become questionable as employees desperately tried to preserve their jobs. Most companies now realize that it is a tool but is not an omnipotent entity. The live production veterinarian must be knowledgeable in all areas of poultry production and management and must keep quite a contact list of experts in all these areas to call upon for assistance. They must balance the company politics of grower management, nutrition and milling, hatchery and live op supervision and maintain their assigned live production costs by doing the best job possible with those factors they can control. No wonder that many young veterinarians are snubbing these jobs in favor of those with more reasonable expectations and working hours.

Poultry veterinary medicine has been very good to me. I have been able to see the world and do things that I never could have imagined when I was in a mixed practice in southwest Missouri. My Walter Mitty inner self has led me to try almost all disciplines of poultry medicine and perhaps I would have been better off if I had stayed put more but I have rarely regretted making a change to learn something new and face new challenges. When asked, I tell an aspiring poultry veterinarian to find a part of poultry production that they find interesting and to learn all they can about that discipline. The field will become more and more complex as we go forward and to be highly knowledgeable in an area will be desirable. There will be many more challenges, especially socio-economic and perhaps disease related such as avian influenza and Newcastle epidemics. Non-animal origin meats will find a place and become a competition as will more pressure from welfare groups. There will almost certainly be as many changes in the next 35 years as in the past and this will present exciting and challenging opportunities for those who will be retiring in the 2050s. One thing for sure, veterinarians choosing a path in poultry veterinary medicine will find it as interesting and exciting as ever.

TABLE OF CONTENTS

CURRENT STATUS OF ANTIMICROBIAL USE, ANTIMICROBIAL RESISTANCE AND FLOCK HEALTH IN CANADIAN POULTRY

A. Agunos[A], S. Gow[B], D. Léger[A], A. Deckert[A], and R. Reid-Smith[A]

[A]Center for Foodborne, Environmental and Zoonotic Infectious Diseases, Public Health Agency of Canada, Guelph, Ontario
and [B]Saskatchewan, SK
email: agnes.agunos@phac-aspc.gc.ca

SUMMARY

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) monitors trends in AMU and AMR in broiler chicken and turkey flocks. The implications of AMU policy changes on bird health is also monitored. In 2019, (CIPARS) reported a reduction in antimicrobial use (AMU) in broiler chicken flocks that paralleled a decrease in antimicrobial resistance (AMR) (1). In 2020, the initial descriptive data indicates that in both broiler chickens and turkeys, that a decrease in total AMU corresponded with a decrease in multiclass resistant (≥ 3 multiclass resistance) *Escherichia coli* without substantial changes in the percentage of mortality and percentage of flocks with reported disease syndromes. Full statistical assessment of these data still needs to be completed.

MATERIALS AND METHODS

In 2020, 115 broiler chicken flocks and 61 turkey flocks were surveyed at the farm-level where AMU data and samples for bacterial culture/susceptibility testing were collected by a network of 16 poultry veterinary and their producers. In brief, AMU data were summarized and reported using count-based, weight-based and dose-based indicators (2). The focus of CIPARS AMU surveillance is on antimicrobials deemed as medically important to human medicine (3), but nonmedically important antimicrobials such as ionophores, chemical coccidiostats and flavophospholipids are also noted. For AMR, *Escherichia coli, Salmonella* and *Campylobacter* were recovered from the pooled fecal samples (four per flock) and isolates were susceptibility tested using the CMV4AGNF and CMV5AGNF panel developed by the United States National Antimicrobial Resistance Monitoring System (4,4). Trends in the percentages of individual or homologous resistances (e.g., resistance to ceftriaxone, gentamicin, tetracycline) and composite (≥3 multiclass resistance, susceptible isolates) AMR outcomes were determined and summarized. For all AMR outcomes prevalence estimates are adjusted for clustering at the flock level, therefore, the proportion of isolates, which are also provided, do not always equate the percentage provided. This is particularly true for organisms with fewer isolates recovered. Detailed farm sampling, laboratory and analytic methods are described elsewhere 2).

RESULTS AND DISCUSSION

Broiler chickens.

AMU. The percentage of broiler flocks exposed to medically-important antimicrobials decreased by 10% from 76% in 2019 to 66% in 2020. The diversity of antimicrobials decreased over time, consistent with the broiler chicken sector's AMU stewardship action aimed to progressively eliminate the preventive use of antimicrobials. In 2020, there were only two antimicrobials reportedly used for the prevention of necrotic enteritis (bacitracin [57%] and avilamycin [18%]). In terms of milligrams per population correction unit (mg/PCU), the total quantity of antimicrobials decreased by 24% from 142 mg/PCU in 2019 to 108 mg/PCU in 2020. The reduced mg/PCU paralleled the change in dose-based indicators, for example, the total number of defined daily doses using Canadian standards per 1,000 animal-days at risk (nDDDvetCA/1,000 animal-days at risk) (454 to 346 nDDDvetCA/1,000 animal-days at risk; a -24% change).

AMR. For *Escherichia coli* the percentage of susceptible isolates increased by 4% from 34% (194/571) in 2019 to 38% (162/422) in 2020. Correspondingly, resistance to ≥3 antimicrobial classes decreased by 10% from 31% (178/571) in 2019 to 21% (90/422) in 2020. Resistance to ceftriaxone, and tetracycline decreased by 3% and 1% respectively between 2019 (ceftriaxone 7% (42/571) and

tetracycline 39% (224/571) and 2020 (ceftriaxone 4% (18/422), tetracycline 38% (147/422)). However, in 2019 gentamicin resistance was 17% (98/571) which rose by 1% to 18% (74/422) in 2020.

For *Campylobacter*, 50% (42/78) of the 2020 isolates were susceptible to the panel of antimicrobials tested, this is a 14% decrease from 64% (92/142) in 2019. Resistance ≥ 3 antimicrobial classes decreased from 0.7% (1/142) to 0% (0/78) in 2020. Gentamicin resistance stayed consistent at 0% in both 2019 and 2020. Tetracycline resistance increased by 14% between 2019 (27%, (36/142) and 2020 (41%, 28/78). Similarly, ciprofloxacin resistance also increased by 6% between 2019 (23%, 32/142) and 2020 (30%, 19/78).

The *Salmonella* data for 2020 are pending.

Flock health. In 2020, the average percentage of mortality remained stable at 4%. Commonly occurring disease syndromes in broilers, except septicemia, decreased between 2019 and 2020 by 5 to 11%.

Turkeys.

AMU. The percentage of flocks exposed to medically-important antimicrobials decreased by 15% from 65% in 2019 to 50% in 2020. As with broiler chickens the diversity of antimicrobials decreased over time, consistent with the turkey sector's AMU stewardship action aimed to progressively eliminate the preventive use of antimicrobials. Bacitracin (57%) and avilamycin (18%) were also the most frequently used antimicrobials for the prevention of necrotic enteritis. In 2020, a significant rise in bambermycin use was detected (7% in 2019 to 21% in 2020). In terms of mg/PCU, the total quantity of antimicrobials decreased by 54% (from 84 mg/PCU in 2019 to 39 mg/PCU in 2020). A decrease in the nDDDvetCA/1,000 animal-days at risk was also detected (95 to 65 nDDDvetCA/1,000 animal-days at risk; a -34% change).

AMR. For *Escherichia coli* the percentage of susceptible isolates increased by 6% from 28% (109/393) in 2019 to 34% (75/223) in 2020. Correspondingly, resistance to ≥3 antimicrobial classes decreased by 9% from 28% (109/393) in 2019 to 19% (42/223) in 2020. Resistance to ceftriaxone, gentamicin, and tetracycline decreased by 1.6%, 3% and 7% respectively between 2019 (ceftriaxone 2%, 6/393; gentamicin 11%, 42/393 and tetracycline 61%, 241/393) and 2020 (ceftriaxone 0.4%, 1/223; gentamicin 8%, 18/223; tetracycline 54%, 120/223).

For *Campylobacter*, 35% (31//90) of the 2020 isolates were susceptible to the panel of antimicrobials tested this is a 4% decrease from 39% (85/214) in 2019. Resistance ≥3 antimicrobial classes remained the same at 0%. Gentamicin resistance also stayed consistent at 0% in both 2019 and 2020. Tetracycline resistance increased by 5% between 2019 (43%, (92/214) and 2020 (48%, 42/90) whereas ciprofloxacin resistance decreased by 19% between 2019 (37%, 76/214) and 2020 (18%, 16/90).

The *Salmonella* data for 2020 are pending.

Flock health. In 2020, the average flock mortality remained relatively stable at 6%. Commonly occurring disease syndromes in turkeys decreased between 2019 and 2020, but increased for yolk sac infections by 6% and miscellaneous bacterial diseases (e.g., clostridial dermatitis) increased by 5%.

CONCLUSION

Ongoing surveillance for AMU at the farm level indicated that quantity of antimicrobials decreased and fewer antimicrobial classes were being used for prevention of these diseases. Increased percentage of *E. coli* isolates susceptible to all the antimicrobials tested, decreased percentage of ≥ 3 multiclass resistant and ceftriaxone resistant *E. coli* indicated that *E. coli* populations in the two poultry species continued to shift towards reduced AMR. However, for *Campylobacter*, a decrease in ciprofloxacin resistance was observed only in turkeys but not in broiler chickens, indicating that variations in farm-level factors affecting AMR need to be examined more closely between these species. Mortality and the reported diagnosis of disease syndromes remained stable except in some cases (e.g., yolk sac infections and miscellaneous bacterial diseases in turkeys).

REFERENCES

1. Huber, L.; Agunos, A.; Gow, S.P.; Carson, C.A.; Van Boeckel, T.P. Reduction in Antimicrobial Use and Resistance to Salmonella, Campylobacter, and Escherichia coli in Broiler Chickens, Canada, 2013-2019. *Emerg Infect Dis* **2021***, 27*, 2434-2444, DOI 10.3201/eid2709.204395 [doi].

2. Government of Canada. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2018: Design and Methods. Available online: https://www.canada.ca/en/public-health/services/surveillance/canadian-integrated-program-antimicrobial-resistance-surveillance-cipars/cipars-reports/2018-annual-report-design-methods.html .

3. Government of Canada. List A: List of certain antimicrobial active pharmaceutical ingredients. Available online: https://www.canada.ca/en/public-

health/services/antibiotic-antimicrobial-resistance/animals/veterinary-antimicrobial-sales-reporting/list-a.html 2020).

4. United States Food and Drugs Administration. NARMS Methodology. Available online: https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/resources (Accessed on October 4 2021).

OPTIMIZATION OF INFECTIOUS BRONCHITIS (IB) VACCINATION STRATEGIES AGAINST EGG PRODUCTION ABNORMALITIES

A. Ali[A,B], M. Hassan[A,C,] S. Gomis[D], A. Shalaby[B], and M. Faizal Abdul-Careem[A,*]

[A]Faculty of Veterinary Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada
[B]Department of Pathology, Faculty of Veterinary Medicine, Beni-Suef University, Beni Suef 62521, Egypt
[C]Department of Poultry Diseases, Faculty of Veterinary Medicine, Assiut University, Assiut 71515, Egypt
[D]Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B5, Canada

* Correspondence: faizal.abdulcareem@ucalgary.ca

INTRODUCTION

Infectious bronchitis is one of the major diseases affecting poultry industry all over the world. Globally, egg production and quality affections resulted from IB have incriminated to induce millions of dollar losses per year. Infectious bronchitis virus (IBV) is a respiratory pathogen belonging to *Coronaviridae* and some IBV strains have been known to cause pathological lesions in the reproductive and respiratory tissues (1). It was shown that IBV could replicate in the ciliated epithelial cells of different parts of chicken oviduct such as magnum, tubular shell gland, and shell gland pouch (1). Occurrence of pathology in these epithelial cells during IBV infection could result in the chickens to produce eggs with shell abnormalities. Multiple studies revealed the deleterious effects of IBV on egg-shell quality (2,3,4). Decreased egg production during IBV infection is varied from 3 to 10 %, however in some instances, it reaches up to 50% (5). Furthermore, IBV recovered chickens may continue to lay fewer eggs with 10 % lower than normal expected values along the laying period (5).

Since 2010, it has been reported that many layer flocks suffered from transient production of shell-less eggs in western Canada (Saskatchewan, Alberta, and Manitoba) (6). The outbreaks among these layer flocks were sporadic, in addition they showed variable ages and produced shell-less eggs. This condition called shell-less egg syndrome (SES) (6); it could be attributed to some strains of IBV that have a tropism to shell gland where shell is synthesized (2). IBV isolates from SES cases have been molecularly characterized into Mass type IBV (6), therefore, it was necessary to employ and optimize a vaccination regime against the identified IBV isolate (Mass type IBV) in layer chickens.

MATERIAL AND METHODS

Chickens. SPF White Longhorn chicks were obtained from Canadian Food Inspection Agency (CFIA), Ottawa, Ontario. Afterwards, the chicks were sexed, and SPF female chicks (n=50) were reared at the Veterinary Science Research Station (VSRS) facility at the Spy Hill campus, University of Calgary.

Vaccines. Two live attenuated IB vaccines were used in this study; one of them contained Mass serotype (Merial INC, Athens, GA, USA), the second had both Mass and Conn serotypes (Zoetis Inc, Kalamazoo, MI, USA). Inactivated IB vaccine containing Mass serotype (Merck Animal Health, Division of Intervet. INC, Omaha, NE, USA) was administrated.

Challenge virus. Mass IBV (characterized as 15AB-01) was used in the present work, and it was isolated from layer flocks with history of SES in Western Canada (6). Virus propagation was carried out via inoculation in the allantoic cavity of SPF embryonated chicken eggs (9- to 11- day- old) at 37 °C for 48 hours. Calculation of chicken embryo infectious dose (EID50) was performed according to Reed and Muench method (7).

Experimental design and sample collection. After sexing of chicks, SPF female chicks were housed and divided into vaccinated group (n= 36) and mock vaccinated group (n=14) till 14 weeks old. Afterwards, the growing pullets in the vaccinated group was divided into one group (n=18) treated with live attenuated vaccine only and the other group (n=18)

vaccinated with both live attenuated and inactivated. During pullet growing period, blood samples were collected at 2-, 5-, 9-, 14- and 17-week-old for serum IBV antibody titers measurement. Just before the peak of lay, the three treated groups were divided into six groups as follow:

a- Non-vaccinated- non challenged group (Con.) (n=5)
b- Live vaccinated- non- challenged group (L) (n=8)
c- Killed and Live vaccinated- non- challenged group (K) (n=8)
d- Killed and Live vaccinated- challenged group (KC) (n=10)
e- Live vaccinated- challenged group (LC) (n=10)
f- Non- vaccinated – challenged group (NVC) (n=9)

At the peak of lay (26-week-old), the treated groups (KC, LC, NVC) were challenged with 200 µl of 1 X 10^6 EID_{50} Mass IBV, designated as 15AB-01 via oculo-nasal route. On the other hand, the other groups (Con., L, K) were inoculated with a placebo (200 µl of phosphate-buffered saline via oculo-nasal route). After challenge, all treated groups were observed for clinical signs, egg production and quality for 14 days post-infection (dpi). Blood samples for detection of serum IBV antibody titers were obtained at 10 and 14 dpi. At 14 dpi, the whole hens were humanely euthanized, and post-mortem examination was done to detect any gross lesions. Furthermore, Tissue samples from trachea, lung, kidney, and portions from oviduct (magnum, isthmus, uterus) were collected as well. The later tissue samples were kept in 10% neutral-buffered formalin (VWR International, Edmonton, AB, Canada) for histopathology.

RESULTS

Regarding the serological screening before the laying period, no anti-IBV antibody titer has been detected in the mock vaccinated group. The mean titer was higher in vaccinated group at 5,9-, and 14-week-old in comparison with the non-vaccinated group. No substantial difference could be detected between killed-live vaccinated group and live vaccinated group at 17-week-old.

In terms of clinical signs post-challenge, only chickens in the non-vaccinated challenged group exhibited respiratory distress starting from 3dpi, but the signs were subsided at 10dpi. The mean clinical scores of the non-vaccinated challenged group were higher from 4dpi till 6dpi in comparison with the other groups. The peak of severity of clinical signs was observed at 5dpi in the non-vaccinated challenged group. All treated groups showed no significant drop in egg production from challenge till the end of experiment. Some egg quality disorders such as thin-shelled eggs and watery albumen were recorded among some hens in NVC group at 6 and 8dpi. Only watery albumen could be observed in one hen belonging to LC group at 12dpi. The mean serum anti-IBV antibody titer was higher in chickens of KC group in comparison with those of NVC group at 10 dpi. The antibody titer was higher in the two vaccinated - challenged groups (KC, LC) in comparison with the vaccinated-mock challenged ones (K,L). The mean titer was higher in all treated groups except Chickens in group L in comparison with the mock vaccinated - mock challenged group. At 14 dpi, there was a difference between the vaccinated- challenged groups (KC, LC) and vaccinated-mock challenged groups (K, L), and the mock vaccinated-mock challenged as well.

During post-mortem examination, there were no gross lesions observed in all groups. Microscopically, no pathological lesions could be observed in different parts of oviduct of control groups (Con, L, K) and all hens in KC group as well. The microscopic lesions were prominent in some birds of NVC group in which there were attenuated and sloughed epithelia with ciliary losses. Furthermore, the lamina propria was characterized by edema, mononuclear cell infiltration and glandular dilatation. Few birds in LC group showed thinner epithelium with patchy deciliation, and low number of inflammatory cells in subepithelial tissue.

DISCUSSION AND CONCLUSION

In the present study, the protective efficacy against challenge with Mass type IBV (15AB-01) in layer chickens was performed by using a vaccination programme composed of a primed dose of monovalent live attenuated vaccine (containing Mass serotype) followed by more than one dose of bivalent live vaccine (containing Mass and Conn serotypes). Additionally, the inactivated vaccine was administrated before the laying period. It has been reported that the Mass type IBV isolate, 15AB-01 was associated with drop in egg production and SES in SPF chickens (6), however we recognized no decline in egg production in all challenged groups. In accordance with our findings, Chousalkar and Roberts (8) reported neither decline in egg production nor deleterious effects in egg quality. The vaccination regime either by live attenuated vaccine alone or a combination of a

live and inactivated vaccines could eliminate the respiratory illness unlike to the results obtained by De Wit et al (9). The later study showed no protection against the respiratory signs in the challenged birds regardless the vaccination programme. Absence of egg quality disorders, higher serum anti-IBV antibody levels accompanied with very minimal microscopic lesions observed among oviduct of chickens in KC group could reflect better protection against IBV challenge in comparison with using live attenuated vaccine alone, thus has been reported by several studies (10, 11, 12).

In conclusion, the present work reveals that Mass type IBV, designated as 15AB-01 is associated with some deleterious effects in mock vaccinated layer chickens inducing respiratory distress, microscopic lesions in oviduct, and abnormalities in egg quality as well. Vaccination regime that composed of a multiple priming doses of heterologous live attenuated vaccine followed by administration of inactivated vaccine could provide a significant protection against Mass IBV challenge in layers.

REFERENCES

1. Chousalkar KK, Roberts JR. Ultrastructural study of infectious bronchitis virus infection in infundibulum and magnum of commercial laying hens. *Vet. Microbiol*. 122: 223–236; 2007. doi: 10.1016/j.vetmic.2007.01.021

2. Sevoian M, Levine PP. Effects of infectious bronchitis on the reproductive tracts, egg production and egg quality of laying chickens. *Avian Dis.* 1:136–164; 1957.

3. Cook JKA. Recovery of infectious bronchitis virus from eggs and chicks produced by experimentally inoculated hens. *J. Comp. Pathol.* 81:203–211; 1971. doi: 10.1016/0021-9975(71)90093-4

4. Munner MA, Halvorson DA, Sivnandan V, Newman JA, Coon CN. Effects of Infectious bronchitis virus (Arkansas strain) on laying chickens. *Avian Dis.* 30(4):644–647; 1986.

5. Bisgaard, M. The influence of infectious bronchitis virus on egg production, fertility, hatchability, and mortality rate in chickens. *Nord. Vet. Med.* 28: 368–376; 1976.

6. Amarasinghe A, Popowich S, de Silva Senapathi U, Abdul-Cader, MS, Marshall F, van der Meer F, Cork SC, Gomis S, Abdul-Careem MF. Shell-Less Egg Syndrome (SES) Widespread in Western Canadian Layer Operations Is Linked to a Massachusetts (Mass) Type Infectious Bronchitis Virus (IBV) Isolate. *Viruses*. 10 (8): 437; 2018. https://doi.org/10.3390/v10080437

7. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints12. *Am. J. Epid.* 27(3): 493-497; 1938. doi.org/10.1093/oxfordjournals.aje.a118408

8. Chousalkar KK, Roberts JR. Ultrastructural Observations on Effects of Infectious Bronchitis Virus in Eggshell-Forming Regions of the Oviduct of the Commercial Laying Hen. *Poult. Sci.* 86:1915–1919; 2007. doi: 10.1093/ps/86.9.1915

9. De Wit JJ, Boelm GJ, van Gerwe TJWM, Swart WAJM. The required sample size in vaccination- challenge experiments with infectious bronchitis virus, a meta-analysis. *Avian Pathol*. 42: 9–16; 2013. doi: 10.1080/03079457.2012.751485.

10. De Wit JJS, Malo A, Cook JKA. Induction of IBV strain-specific neutralizing antibodies and broad-spectrum protection in layer pullets primed with IBV Massachusetts (Mass) and 793B vaccines prior to injection of inactivated vaccine containing Mass antigen. *Avian Pathol.* 48:135–147; 2019. doi:10.1080/03079457.2018.1556778.

11. De Wit JJS, Ter Veen C, Koopman HCR. Effect of IBV D1466 on egg production and egg quality and the effect of heterologous priming to increase the efficacy of an inactivated IBV vaccine. *Avian Pathol.* 49:185–192; 2020. doi: 10.1080/03079457.2019.1710462.

12. Box P, Ellis KR. Infectious bronchitis in laying hens: Interference with response to emulsion vaccine by attenuated live vaccine. *Avian Pathol.* 14:9–22; 1985. doi: 10.1080/03079458508436204.

INFECTIOUS LARYNGOTRACHEITIS HVT RECOMBINANT CONSTRUCTS: REPLICATION AND EFFICACY AGAINST CHALLENGE IN BROILERS

I. Alvarado[A], D. Maekawa[B], and M. García[B]

[A]Merck Animal Health, Madison, NJ, USA
[B]Poultry Diagnostic and Research Center, University of Georgia, Athens, GA, USA

SUMMARY

The efficacy of several commercial recombinant HVT vaccines (rHVT) expressing glycoproteins of infectious laryngotracheitis against a virulent infectious laryngotracheitis virus (ILT) challenge in broilers were compared. Efficacy was evaluated based on their capacity to reduce clinical signs, decrease viral genome loads of the challenge strain in tracheas post-challenge and survival rates. When compared with the non-vaccinated/challenged group (NVx/Ch) all the evaluated vaccines provided protection against challenge, decreasing clinical signs and replication of the challenge strain. However, differences in their efficacy were observed. Overall, considering the conditions of this study and criteria used to evaluate their efficacy, one of the single (rHVT-ILT) and the dual (rHVT-IBD-ILT) vaccines offered superior protection against ILT in broilers challenged at 21 and 40 days of age.

INTRODUCTION

Infectious laryngotracheitis (ILT), caused by Gallid alpha-herpesvirus -1 (GaHV-1), is a highly contagious respiratory disease of chickens that produces significant economic losses to the poultry industry (1,6). Control of the disease is based on strict biosecurity and the implementation of vaccination programs in affected areas (7).

Live commercial vaccines have been successfully used to control outbreaks. However, their capacity to spread from bird to bird regaining virulence, particularly the chicken embryo vaccines, and the establishment of latent infections with sporadic reactivation leading to virus shedding are a matter of concern in the control of the disease (2,3,5,8). As a response to the frequent ILT epizootics related to CEO vaccines, a new generation of recombinant vaccines using fowl poxvirus and herpesvirus of turkey (HVT) as vectors were developed (7). Currently, HVT vectored vaccines (rHVT) expressing one (B) or two (I and D) glycoproteins of ILT virus are commercially available. rHVT-ILT vaccines are characterized by persistent viremia and the ability of the HVT vector to replicate in lymphocytes in a highly cell-associated manner, establishing long lasting cell mediated immunity (4).

OBJECTIVE

The objective of this study was to compare the level of HVT replication in feather follicles and protection provided by commercially available rHVT-ILTV vaccines in broiler-type chickens against a virulent infectious laryngotracheitis challenge.

MATERIALS AND METHODS

Vaccines and treatment groups. A total of one hundred fifty 14-day-old fertile embryos were obtained from a commercial broiler flock and incubated at 99.5°F and 55% relative humidity (RH) in a small-scale hatcher (Natureform Inc., Jacksonville, FL) at the Poultry Diagnostic Research Laboratory (PDRC). At 18.5 days of embryonation, eggs were randomly divided in five groups of 30 eggs each. Three groups were manually vaccinated *in ovo* using a full dose of each of the following vaccines:

1. rHVT-ILT expressing glycoproteins I and D
2. rHVT-ILT(2) expressing the glycoprotein B
3. rHVT-IBD-ILT expressing the glycoprotein D

The remaining two groups, non-vaccinated/non-challenged (NVx/NCh) and non-vaccinated/challenged (NVx/Ch), were mock inoculated *in ovo* with Marek's vaccine diluent. Accuracy during manual *in ovo* vaccination was evaluated in additional embryos by injecting Marek's diluent with a blue dye.

Detection of HVT genome post-vaccination. Feather follicle material was collected from broilers from all the vaccinated groups and the non-vaccinated group at 14 days post-vaccination. Total DNA was extracted, with the presence of the HVT vector detected by real-time PCR and viral genome load expressed as the $\log_{10} 2^{-\Delta\Delta Ct}$. Broilers in the vaccinated groups negative for the detection of the HVT genome were removed from the study.

Challenge. At 21 and 40 days of age, 16 and 10 broilers from each of the vaccinated and the NVx/Ch groups, respectively, were challenged with the highly pathogenic ILTV virulent strain (1874C5) at a dose of $10^{3.8}$ $TCID_{50}$. The challenge virus was administered in a total volume of 200 µL split into 50 µL delivered in each eye, and 100 µL delivered intra-tracheally. The remaining non-vaccinated/non challenged (NVx/NCh) group was mock inoculated with tissue culture media.

Efficacy. Vaccine protection was estimated through reduction of clinical signs of the disease and reduction of challenge virus replication in the trachea. From three to six days post-challenge each bird received a clinical sign score (0- 3) for signs of conjunctivitis, dyspnea, and lethargy. Briefly, normal: 0, mild: 0.5-1, moderate: 1.5-2, and severe: 2.5-3. Any mortality received a score of six. The total clinical signs score per chicken was estimated and the mean clinical sign score per vaccinated group per time point was reported.

Challenge virus genome load from tracheal swabs post-challenge was determined. Briefly, tracheal swabs were collected from vaccinated and non-vaccinated birds at days three and five post-challenge and individually placed in 1.5 mL microcentrifuge tubes. Samples were stored at −80°C until processing. Total DNA was extracted, and real-time PCR was used to estimate the genome viral load of the challenge virus. Individual and average genome load were expressed as the $\log_{10} 2^{-\Delta\Delta Ct}$.

RESULTS

Detection of HVT genome post-vaccination. The presence of the HVT vector was detected in 100%, 95% and 100% of the broilers vaccinated with the rHVT-ILT, rHVT-ILT (2) and rHVT-IBD-ILT vaccines, respectively. Mean viral genome load values of 4.1, 4.1 and 3.3 were detected in broilers vaccinated with the rHVT-ILT, rHVT-ILT(2) and rHVT-IBD-ILT vaccines, respectively.

Mean clinical signs. Mean clinical signs scores from three to six days post-challenge in broilers challenged at 21 or 40 days with the virulent 1874C5 ILT strain and the non-vaccinated/non challenged group are summarized in Table 1.

Vaccinated broilers challenged at 21 days exhibited a peak of clinical signs four days post-challenge. Even though all the vaccinated groups showed a reduction in mean clinical signs, scores differences among groups were observed. Broilers vaccinated with the rHVT-ILT and rHVT-IBD-ILT showed a significant reduction in clinical signs post-challenge when compared with the non-vaccinated/challenged group. In contrast, no significant differences in clinical signs were observed at four- and five-days post challenge between broilers vaccinated with the rHVT-ILT(2) and the non-vaccinated/challenged group. As expected, no clinical signs were present in the non-vaccinated/non-challenged group. Protection against mortality was also variable among groups. Survival rates at six days post-challenge of 80%, 70%, 100%, and 35% were observed in the rHVT-ILT, rHVT-ILT (2), rHVT-IBD-ILT and the NVx/Ch groups, respectively.

Clinical signs post-challenge in vaccinated broilers challenged at 40 days showed a similar pattern, with peak of clinical signs at four days of age and lower clinical signs scores in the vaccinate groups when compared with the non-vaccinated/challenged group. The lowest clinical signs were observed in the rHVT-ILT vaccinated group, followed by the rHVT-IBD-ILT and rHVT-ILT (2) groups. Survival rates at six days post-challenge of 100%, 58%, 100%, and 0% were observed in the rHVT-ILT, rHVT-ILT(2), rHVT-IBD-ILT and the NVx/Ch groups, respectively.

Challenge virus genome load. Mean genome viral load of the 1874C5 ILT strain in tracheal swabs from broilers challenged at 21 and 40 days of age is summarized in Table 2. Viral load is expressed as the $Log_{10}^{\Delta\Delta Ct}$ values at three- and five-days post-challenge. When viral genome loads at three- and five-days post-challenge were compared, a significant reduction was observed in all the vaccinated groups and the non-vaccinated/challenged group.

In broilers challenged at 21 days, the lowest genome levels among the vaccinated groups were detected at three- and five-days post-challenge in broilers vaccinated with the rHVT-ILT vaccine. Furthermore, at five days post-challenge, no significant differences in viral genome loads were observed between the rHVT-ILT vaccinated and non-vaccinated/non-challenged group.

In broilers challenged at 40 days of age, significantly lower viral genome loads were observed at three days post-challenge in the rHVT-ILT group

when compared with the rHVT-IBD-ILT group. At five days post-challenge, no significant differences were observed among the vaccinated groups and the non-vaccinated/challenged group.

REFERENCES

1. Davison, A. J., R. Eberle, G. S. Hayward, D. J. McGeoch, A. C. Minson, P. E. Pellet, B. Roizman, M. J. Studdert, and E. Thiry. The order Herpesvirales. Arch. Virol. 154:171-177. 2009.

2. Guy, J. S., H. J. Barnes, and L. M. Morgan. Virulence of infectious laryngotracheitis viruses: comparison of modified-live vaccine viruses and North Carolina field isolates. Avian Dis. 34:106-113. 1990.

3. Guy, J. S., H. J. Barnes, and L. Smith. Increased virulence of modified-live infectious laryngotracheititis vaccine virus following bird-to-bird passage. Avian Dis. 35:348-355. 1991.

4. Heller, E. D., and K. A. Schat. Enhancement of natural killer cell activity by Marek's disease vaccines. Avian Pathol. 16:51-60. 1987.

5. Hughes, C. S., R. C. Jones, R. M. Gaskell, F. T. Jordan, and J. M. Bradbury. Demonstration in live chickens of the carrier state in infectious laryngotracheitis. Res. Vet. Sci. 42:407-410. 1987.

6. Maekawa, D., G. Beltrán, S. M. Riblet, and M. García. Protection efficacy of a recombinant herpesvirus of turkey vaccine against infectious laryngotracheititis virus administered in ovo to broilers at three standardized doses. Avian Dis. 63:351-358. 2019.

7. Maekawa, D., S. M. Riblet, L. Newman, R. Koopman, T. Barbosa, M. Garcia. Evaluation of vaccination against infectious laryngotracheitis (ILT) with recombinant herpesvirus of turkey (rHVT-LT) and chicken embryo origin (CEO) vaccines applied alone or in combination. Avian Path. 48(6): 573-581. 2019.

8. Menendez, K. R., M. García, S. Spatz, and N. L. Tablante. Molecular epidemiology of infectious laryngotracheitis: a review. Avian Pathol. 43:108-117. 2014.

Table 1. Mean clinical signs scores from three to six days post-challenge in vaccinated and non-vaccinated broilers challenged at 21 or 40 days of age with the virulent 1874C5 ILT strain.

		Treatment Groups				
	Days Post-Challenge	rHVT-ILT	rHVT-ILT (2)	rHVT-IBD-ILT	NVx/Ch	NVx/NCh
21 Days Challenge	3	1.1^{b}	1.5^{b}	1.1^{b}	3.4^{c}	0.0^{a}
	4	2.2^{b}	5.1^{c}	2.0^{b}	6.0^{c}	0.0^{a}
	5	1.0^{b}	3.4^{c}	1.2^{b}	5.1^{c}	0.0^{a}
	6	0.4^{a}	1.5^{b}	0.5^{a}	2.9^{c}	0.0^{a}
40 Days Challenge	3	0.5^{a}	3.0^{b}	0.9^{a}	4.6^{c}	0.1^{a}
	4	0.7^{a}	3.7^{b}	$1.4^{a,b}$	6.1^{c}	0.1^{a}
	5	$0.7^{a,b}$	1.9^{b}	$0.7^{a,b}$	6.0^{c}	0.1^{a}
	6	0.1^{a}	1.6^{b}	$0.8^{a,b}$		0.1^{a}

Table 2. Mean HVT genome detected in tracheal swabs from broilers challenged at 21 or 40 days of age with the virulent 1874C5 ILT strain and the non-challenged control group. HVT genome load, detected at three- and five-days post-challenge, is expressed as $Log_{10} 2^{-\Delta\Delta}$ of Ct values.

		Treatment Groups				
	Days Post-Challenge	rHVT-ILT	rHVT-ILT (2)	rHVT-IBD-ILT	NVx/Ch	NVx/NCh
21 Days Challenge	3	3.4^{b}	4.9^{c}	4.8^{c}	5.2^{c}	0.0^{a}
	5	0.8^{a}	2.6^{b}	2.0^{b}	3.1^{b}	0.0^{a}
40 Days Challenge	3	4.1^{b}	$4.9^{b,c}$	4.9^{c}	$5.0^{b,c}$	0.0^{a}
	5	3.0^{b}	2.2^{b}	3.0^{b}	3.3^{b}	0.0^{a}

CAMPYLOBACTER HEPATICUS IN THE PRODUCTION ENVIRONMENT/STAGNANT WATER AS A POTENTIAL SOURCE OF CAMPYLOBACTER CAUSING SPOTTY LIVER DISEASE IN FREE RANGE LAYING HENS IN GEORGIA, USA

R. Becerra, J. Nicholds, N. Barbieri, C. Nakatsu, K. Grogan, D. French, E. Shepherd, and C. Logue

Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602

SUMMARY

Spotty liver disease (SLD) has emerged as an important cause of disease in layer (egg-producing) birds in countries such as the United Kingdom and Australia. In the USA, SLD has been found in the Midwest and Southeast. The organism implicated in the disease, *Campylobacter hepaticus*, causes focal lesions on the livers of infected birds, reduced egg production, decreased feed consumption resulting in reduced egg size, and increased mortality of highly valuable hens. In the fall of 2021, birds from two flocks A and B of organic pasteurized laying hens were submitted to the Poultry Diagnostic Research Center at the University of Georgia (PDRC, UGA) to diagnose possible SLD in the flocks. Necropsy of flock A, 5/6 hens had multifocal foci lesions on the liver and showed PCR positive for *C. hepaticus* on pooled swab samples of liver and gall bladder. Necropsy of flock B, 6/7 submitted birds had spotty liver lesions. Pooled bile swabs samples from two live hens from flock B were PCR positive for C. *hepaticus*. Follow-up visits to flock A and a flock where SLD has not been reported (flock C) was used as a comparative control. Samples of the liver, spleen, cecal tonsil, ceca, blood, and gall bladder from 6 hens per house, as well as feed, water nipple swaps, and environmental water (stagnant water outside the house), were collected from the affected farm and the control farm. To detect the organism, all samples were subjected to enrichment in Preston Broth and culture under microaerobic conditions. After multiple phases of bacterial culture purification from all samples, single bacterial cultures displaying characteristics of *C. hepaticus* were tested with PCR to confirm identity. Flock A, liver, cecal, cecal tonsils, gall bladder, and environmental water were PCR positive for *C. hepaticus.* Interestingly, flock C, where there have not been reports of *C. hepaticus,* found that the feces, liver, spleen, cecal, gall bladder, and environmental water were also PCR positive for *C. hepaticus.* After another follow-up visit, ten weeks later, flock A was again PCR positive for C. *hepaticus* only in gall bladder bile; however, flock C was PCR negative this time for C. *hepaticus*. Currently, there are no approved treatments, and no vaccine is available for *C. hepaticus.* These results suggest that *C. hepaticus* may be endemic in some areas of the USA, and free-range laying hens may be exposed from the environment/stagnant water in areas where they range. Nevertheless, the pathophysiology and how hens are contracting *C. hepaticus* and if it is endemic are still not well understood, warranting further investigation.

INTRODUCTION

Spotty liver disease (SLD) is a disease that has been identified for decades. When spotty liver disease appeared in 1950, it was known as avian vibronic hepatitis. At that time, no one was able to identify the cause. In 2015, Crawshaw *et al*. identified a novel *Campylobacter* strain (2). Nevertheless, Van *et al*. proposed the name *Campylobacter hepaticus* and demonstrated that *C. hepaticus* was the cause of SLD by reproducing the disease (10,11). *Campylobacter hepaticus* is a Gram-negative organism that grows under microaerobic conditions at 37° to 42° C, has an S-shape, and single bipolar flagella. The colony morphology shows cream-colored, flat-spreading wet colonies.(11). Since live bacteria have been isolated from feces collected from flocks affected with *C. hepaticus*, the infected route is most likely fecal-oral (7,12). *Campylobacter hepaticus* appears to affect hens at peak production but has been reported in birds as young as 25-26 weeks of age (7). The pathophysiology of SLD is not well known; however, what is known is that *C. hepaticus* is primarily found in birds housed in free-range housing systems, although reports have also documented morbidity due to *C. hepaticus* in layers caged under poor hygienic conditions (9,11). Spotty liver disease is an emerging disease in the USA, Jordan, Australia, New Zealand,

the United Kingdom, and probably in other parts of the world where layer hens are kept in a free-range environment (1,3–5). With the increase of organic egg production and animal welfare, more layer hens are kept in a free-range environment, and these hens have the option to utilize all the space given outside the house. Allowing hens to go outside the houses increases the risk of exposure to diseases that can be carried by wild animals or by consuming contaminated water found in puddles outside the house made during rain events. An epidemiological study of SLD in Australia found that environmental sources could be potential sources of *C. hepaticus* transmission (7). Once hens are allowed to go outside, it is not easy to control what they eat or drink outside the houses. When *C. hepaticus* infects hens, gross lesions are characterized by multifocal small round white foci on the liver surface (10). Currently, there are no approved treatments for SLD in organic flocks, but companies may try to acidify the water with apple cider vinegar, oregano, citric acid, and treat with CTC (Aureomycin) if permitted. SLD is a disease that may occur year-round, and further outbreaks within the same flock when birds are in peak production are possible (7). Novel treatments such as isoquinoline alkaloids induce partial protection of laying hens affected by SLD (8). In addition, biochar fed to birds can lower *Campylobacter hepaticus* and may decrease SLD in layer hens (13). From personal experience, producers, or growers in the USA, often see SLD during the wet and hot season, and once a flock is infected, the next flock will likely contract the disease even after a good house cleanout and appropriate downtime.

MATERIALS AND METHODS

Flock A. Six-layer hens, 66 weeks of age, Lohman Brown breed from were submitted to PDRC UGA with the complaint of high mortality and decreased feed consumption. The company reported seeing spotty livers on-field necropsy and treated the flock with oregano and apple cider vinegar. Hens sent for necropsy were randomly picked up from the farmhouse. On presentation, all layer hens looked normal with no signs of illness. Hens were humanly euthanized, liver samples were collected for histopathology, bile and liver swabs were collected for *C. hepaticus* PCR testing.

Flock B. Three dead layers hens and four live hens, 30 weeks of age, Lohman Brown breed were submitted to PDRC UGA with the presenting complaint of high mortality and 15% lost production. Two hens died before they were humanely euthanized for necropsy. The remaining hens were humanly euthanized, and three bone marrow samples were collected for traditional bacteriology culture. Also, pooled bile samples were collected from live and dead hens for *C. hepaticus* PCR.

Flock C. This flock was used as a negative control for the follow-up visits. Six-layer hens, 28 weeks of age, Lohman Brown breed without a history of SLD.

Follow-up visits to flock A and C. On a follow-up visit to flock A and C, flock A has a history of SLD, while flock C does not have a history of SLD. It was raining when flock A and C were visited, and hens were not allowed to go outside for the past two days due to the rain. In flock A and C, three hens out of production and three healthy birds were selected for necropsy. The collected samples included stagnant water outside houses where the flocks were housed, swabs from water drinkers, feces, feed, liver, spleen, gall bladder, and bile and cecal tonsils. Swabs, feed, feces, blood, and stagnant water samples were collected from different house parts. The stagnant water samples were collected from different areas outside the house. All samples were placed on ice just after being collected. All samples collected were labeled according to the flock number and bird number they originated. Liver samples from flock A only were collected for histopathology.

Ten weeks later, a follow-up visit to flocks A and C were performed to collect additional samples. On the sampling week, the weather was pleasant, with no rain on the previous two days, and birds were allowed to go outside each day. Three hens were out of production in flock A and C, and three healthy hens were selected for necropsy. The collected samples included feces, liver, bile, and ground wet soil in these two houses. Feces and samples were collected from different parts of the house. Ground wet soil was collected from different areas outside the house. All samples were placed on ice after collection. On arrival at the lab, all samples were labeled according to the farm number and bird number where they originated. Liver samples from both flocks were collected for histopathology.

Samples for analysis. Over 100 samples were collected from flock A and C. These included swabs of nipple waterers, feed, feces from the ground, liver, spleen, ceca and cecal tonsils, blood, bile, soil, and stagnant water samples from the farm. All samples were logged, assigned an ID and portions of samples placed in 1 ml of Preston enrichment broth with incubation of the broth at 37°C for up to 7d. At day 2 and 7d enrichment, the enrichment broths were struck

to duplicate blood agar plates with one set of plates incubated at 37°C for 7d and the second set incubated at 42°C for 7d. Plates were checked for growth at 3d incubation and at 7d to identify any potential suspect cultures. All enrichment broths and the blood plates were incubated under microaerophilic conditions using Mitsubishi Anaero-MicroAero gas pouches.

For bile samples, a 30µl volume of the bile was plated directly on duplicate plates, struck out and incubated at 37 and 42°C under microaerobic conditions for 7d. Plates were checked at 3 and 7d for suspect growth. Following incubation, all plates were inspected for colonies typical of *Campylobacter* morphology and were selected for PCR analysis.

DNA extraction and analysis. Suspect *Campylobacter* isolates were picked and added to 100µl of sterile water in a 200µl tube. Then the suspensions were prepared using the boil prep method (heating the suspension to 100°C for 10 minutes followed cooling to 10°C for 10 minutes). The suspension was then centrifuged to precipitate cellular debris and the supernatant (containing DNA) removed to a new tube for analysis.

PCR analysis was carried out using primers targeting the glycerol kinase gene, primers G2F3: CAGGAGTTTTACCACAATTC, G2R2: CAAGCTAAAACAGGTTTGG, Tm: 56, and Amplicon size (bp): 463 (12). PCR amplification was carried out in a 25ul reaction consisting of water 18.725µl, PCR buffer 2.5µl, DNTP 1.25µl, primers (0.2 µl) TAQ 0.125 µl and DNA 2 µl. Positive controls included *C. hepaticus* NCTC 13823 (HV10) as the positive control and water in place of DNA for the negative control.

Amplification was carried out on an Eppendorf X50 thermocycler using the following parameters 98°C for 1 minute followed by 35 cycles of 98°C for 10 s; 57°C for 30s and 72°C for 30s with a final extension of 72°C for 10 minutes. All PCR products were subjected to gel electrophoresis on a 1.5% agarose gel in TAE at 100V for 60 minutes.

Following electrophoresis, the gel was stained in 0.25% ethidium bromide for 20-30 minutes and PCR products visualized under UV light using an imager (Aplegen).

RESULTS

Flock A. This farm has a history of SLD. They had increased mortality and decreased production. On this farm, birds have been treated with oregano in the water for the past two days. Hens submitted to the necropsy lab, 5/6 hens had spotty liver lesions (Figure 1-A), and a few had round and ceca worms. This flock was PCR positive for *C. hepaticus* on pooled swab samples of liver and gall bladder. Histopathology on the liver showed subacute mild to moderate multifocal necrotizing and fibrinolymphocytic or heterohistiocity hepatitis (Figure 2-B). Also, there was a subacute, mild to moderate multifocal cholangiohepatitis with biliary hyperplasia and rupture (Figure 2-A). According to the histopathologist who read and gave the morphologic diagnosis to all the slides on this project (Dr. Susan Williams), changes in the liver suggest an ascending or hematogenous infection possible due to bacteria. On the first follow-up visit, 3/6 birds had mild spotty liver lesions during necropsy, and 1/6 had pale liver with multiple pale foci and petechial hemorrhage. After multiple phases of bacterial culture purification from all samples, single bacterial cultures displaying characteristics of *C. hepaticus* were tested with PCR to confirm identity. Of all samples tested, *C. hepaticus* was detected by PCR from enrichment or direct plated samples from flock A of the gall bladder, liver, ceca, feces, stagnant water, and cecal tonsils (Table 1). On the first follow-up visit to this flock, histopathology showed mild to moderate multifocal lymphocytic and fibrous hepatitis. According to the histopathologist, liver changes such as fibrin mixed with an increased population of lymphocytes and macrophages in the parenchyma are suggestive of an infectious agent. On the second follow-up to this farm, 4/6 birds had mild foci liver lesions. In this flock, only bile samples (4/6) were PCR positive for C. *hepaticus*. Histopathology showed an acute multifocal necrotizing hepatitis and moderate multifocal lymphocytic cholangiohepatitis. According to the histopathologist, the changes seen in the liver are consistent with an infectious agent.

Flock B. This flock also had a history of Spotty Liver Disease. Necropsy of flock B, 4/5 submitted dead birds had spotty liver lesions, and 2/2 summited live birds have had spotty livers. Interestingly, pooled swabs samples from the submitted dead hens were PCR negative for C. *hepaticus*, while pooled bile swabs samples from the two live birds were also PCR positive for C. *hepaticus*. Bone marrow samples collected for traditional bacteriology culture were all negative. There was no follow-up visit for this flock, and no samples were sent for histopathology.

Flock C. This farm has no history of SLD. The hens looked healthy. During necropsy, all 6/6 birds looked normal. PCR positive samples for C. *hepaticus* were detected in the gall bladder, liver ceca, cecal tonsil, and stagnant water in the first follow-up visit (Table 1). During necropsy, 6/6 hens

had no gross lesion; therefore, samples for histopathology were not submitted. However, in the second follow-up visit, all the samples were PCR negative for *C. hepaticus* (Table 1). Histopathology showed that 1/5 sections submitted showed moderate to severe, multifocal lymphocytic cholangiohepatitis, suggesting an infectious agent.

DISCUSSION

At the Poultry Diagnostic Research Center at the University of Georgia (PDRC, UGA), the incidence of SLD among submitted cases seems to have increased compared to the previous year. Both flocks A and B submitted to PDRC occurred during fall 2021. To the authors' knowledge, this is the first report of *C. hepaticus* isolation from organic pasteurized laying hens with SLD in Georgia, United States. However, Gregory *et al*., 2018) were the first to report isolation of *C. hepaticus* from Hy-Line W36 breed hens in the midwestern United States in a high-rise cage system (4).

Hens from flock A were 66 weeks of age, while hens from flock B were 30 weeks of age. The spotty liver disease seems to affect hens at peak production and during hot and wet weather climates; however, in this study, *C. hepaticus* seems to affect any age. In an epidemiological study carried out in 2020, Phung *et al*., 2020 found that *C. hepaticus* can infect hens during the rearing phase and before they start to lay eggs with outbreaks of SLD occurring primarily during peak production, but it can also occur before or after the production cycle (7). In this study, both flocks had gross lesions that resemble SLD. Flock A was PCR positive for *C. hepaticus* from pooled bile and liver swabs, and flock B was PCR positive *C. hepaticus* from pooled swabs. Since SLD is an emerging disease in Georgia, USA, and due to challenges growing *C. hepaticus* in culture settings, most laboratories are working to refine the culture method to isolate *C. hepaticus*. This is why PCR was only performed when those two flocks were presented to PDRC.

During presentation, the hens from flock A looked healthy. Nevertheless, when they were opened for necropsy, 5/6 hens had moderate SLD lesions. According to the live production manager, hens were randomly picked up from the house. The liver lesions observed were not extremely severe, but it could be because flock A has been treated with oregano and apple cider vinegar. In addition, liver lesions could decrease or disappear after a couple of days of infection with *C. hepaticus* (10). Although the efficacy of those two treatments has not been evaluated scientifically, some producers believe that it is helping to reduce clinical signs of SLD in organic layer hens where antibiotics are not allowed. However, novel treatments such as isoquinoline alkaloids and biochar could help reduce SLD in layer hens (8,13). When hens from flock B were presented to the necropsy floor, they showed signs of illness. In fact, 2/4 live hens died before they were opened, and 6/7 had SLD lesions. It is not well understood how fast infected hens with *C. hepaticus* die; clinical signs are not always identified because of the instantaneous death of infected hens. In the first follow-up visit, a sick flock (flock A) and a non-sick flock (flock C) were visited. Cultures from samples of the gall bladder, liver, ceca, feces, stagnant water, and cecal tonsils collected from both flocks that showed suspect *Campylobacter* colonies on the plates were run for PCR and were PCR positive for *C. hepaticus*. However, in a second follow-up visit, only flock A was PCR positive for *Campylobacter* in bile/gall bladders and not environmental samples. It is essential to know that some *Campylobacter* species could not be differentiated using culture plates alone; therefore, PCR must be run to confirm *C. hepaticus* in culture and differentiate it from other *Campylobacter* species. It has been shown that *C. hepaticus* can enter a viable but nonculturable state during stress, which can prolong the survival of *C. hepaticus* in the environment and make it difficult to isolate in culture from environmental samples (6). The fact that flock C (negative flock) was PCR positive during the first visit but negative during the second visit could be related to the PCR techniques, and all samples were genuinely negative from flock C, where SLD has never been observed. During the second follow-up visit, culture methods and PCR tests were refined to be more precise in the identification of *C. hepaticus*.

CONCLUSION

With the increase of free-rage rearing, biosecurity plays a vital role in controlling SLD. This includes ensuring good fences to keep wildlife out that could carry not only *C. hepaticus*, but other diseases that can cause disease in hens. In addition, standing water should be avoided reducing the incidence of SLD in free-range hens. These results suggest that *C. hepaticus* may be endemic in some areas of the USA, and free-range laying hens may be exposed from the environment/stagnant water in areas where they range. Nevertheless, the pathophysiology and how hens contract *C. hepaticus* and the endemic status of *C.*

hepaticus are still not well understood, warranting further investigation. Since there are no approved treatments, and no vaccine is available for *C. hepaticus*, further research is necessary to determine the most effective treatment for layer hens affected by SLD.

REFERNCES

1. Crawshaw, T., and R. Irvine. Poultry health: Spotty liver syndrome in poultry in Great Britain. Vet. Rec. 170: 317–318. 2012.

2. Crawshaw, T. R., J. I. Chanter, S. C. L. Young, S. Cawthraw, A. M. Whatmore, M. S. Koylass, A. B. Vidal, F. J. Salguero, and R. M. Irvine. Isolation of a novel thermophilic Campylobacter from cases of spotty liver disease in laying hens and experimental reproduction of infection and microscopic pathology. Vet. Microbiol. 179: 315–321. 2015.

3. Crawshaw, T. R., S. Hunter, D. A. Wilkinson, L. E. Rogers, N. H. Christensen, and A. C. Midwinter. Isolation of Campylobacter hepaticus from free-range poultry with spotty liver disease in New Zealand. N. Z. Vet. J. 69: 58–64. 2021.

4. Gregory, M., B. Klein, O. Sahin, and G. Girgis. Isolation and Characterization of Campylobacter hepaticus from Layer Chickens with Spotty Liver Disease in the United States. Avian Dis. 62: 79–85. 2018.

5. Hananeh, W., and M. Ababneh. Spotty liver disease in Jordan: An emerging disease. Vet. Med. (Praha). 66: 94–98. 2021.

6. Phung, C., R. J. Moore, and T. T. H. Van. Campylobacter hepaticus, the cause of Spotty Liver Disease in chickens, can enter a viable but nonculturable state. Vet. Microbiol. 266: 109341. 2022.

7. Phung, C., B. Vezina, A. Anwar, T. Wilson, P. C. Scott, R. J. Moore, and T. T. H. Van. Campylobacter hepaticus, the Cause of Spotty Liver Disease in Chickens: Transmission and Routes of Infection. Front. Vet. Sci. 6: 1–8. 2020.

8. Quinteros, J. A., P. C. Scott, T. B. Wilson, A. M. Anwar, T. Scott, C. Muralidharan, T. T. H. Van, and R. J. Moore. Isoquinoline alkaloids induce partial protection of laying hens from the impact of Campylobacter hepaticus (spotty liver disease) challenge. Poult. Sci. 100. 2021.

9. Scott, P. C. Determining the cause and methods of control for spotty liver disease." Australian Egg Corporation Limited, North Sydney, NSW. Final Proj. Rep.: 1408. 2016.

10. Van, T. T. H., E. Elshagmani, M. C. Gor, A. Anwar, P. C. Scott, and R. J. Moore. Induction of spotty liver disease in layer hens by infection with Campylobacter hepaticus. Vet. Microbiol. 199: 85–90. 2017.

11. Van, T. T. H., E. Elshagmani, M. C. Gor, P. C. Scott, and R. J. Moore. Campylobacter hepaticus sp. nov., isolated from chickens with spotty liver disease. Int. J. Syst. Evol. Microbiol. 66: 4518–4524. 2016.

12. Van, T. T. H., M. C. Gor, A. Anwar, P. C. Scott, and R. J. Moore. Campylobacter hepaticus, the cause of spotty liver disease in chickens, is present throughout the small intestine and caeca of infected birds. Vet. Microbiol. 207: 226–230. 2017.

13. Willson, N. L., T. T. H. Van, S. P. Bhattarai, J. M. Courtice, J. R. McIntyre, T. P. Prasai, R. J. Moore, K. Walsh, and D. Stanley. Feed supplementation with biochar may reduce poultry pathogens, including Campylobacter hepaticus, the causative agent of Spotty Liver Disease. PLoS One 14: 1–16. 2019.

Figure 1. Picture A shows a hen with spotty liver from flock A presented at the necropsy lab. The yellow circles highlight the white foci necrotizing lesions on the liver. Picture B shows PCR gel electrophoresis. L= 10,000 bp DNA ladder; N = Negative control sample; P = Positive control sample; S = Three positive bile samples (463 bp) from second follow-up visit to flock A.

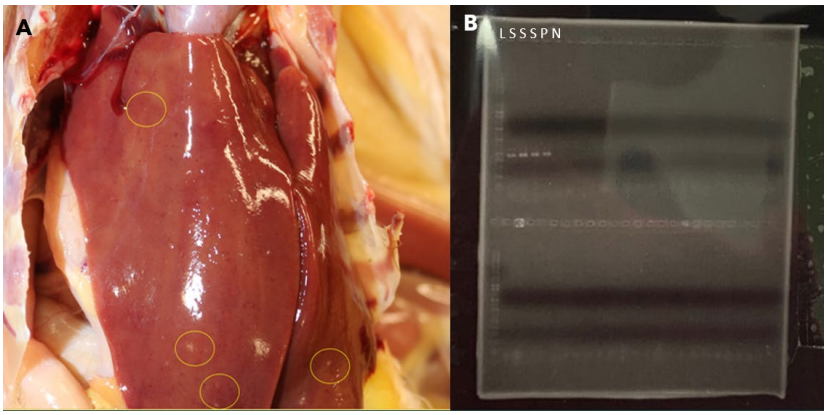


Figure 2. Histopathology slides were from flock A presented at the necropsy lab. Dr. Susan Williams gave morphologic diagnoses from UGA, PDRC. Picture A: Mild to moderate subacute, multifocal cholangiohepatitis has biliary hyperplasia and rupture (yellow circle). Picture B: There is mild to moderate subacute multifocal necrotizing and fibrinolymphocytic or heterohistiocity hepatitis (yellow circle). Changes in the liver suggest an ascending or hematogenous infection possible due to bacteria

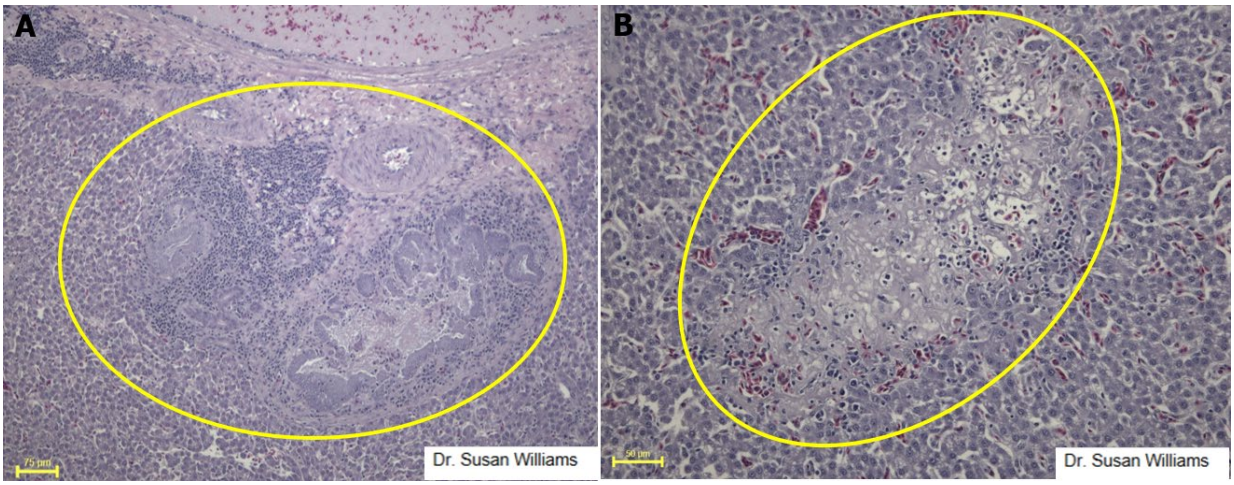


Table 1. PCR results for *C. hepaticus*. Flocks A and C were visited twice for sample collection, but flock B was not. There were at least six swab samples per pooled bile and liver.

C. *hepaticus* PCR results				
Hens and samples summited to the necropsy laboratory				
1 pooled Sample/ category	Flock A		Flock B	
Sample	PCR: +/-	Number of + samples	PCR: +/-	Number of + samples
Pooled bile swabs	+	1	+	1
Pooled liver swabs	+	1		
First follow-up visit for sample collection				
6 samples/category	Flock A		Flock C	
Samples	PCR: +/-	Number of + samples	PCR: +/-	Number of + samples
Nipple swap water				
Feed				
Feces			+	1
Liver	+	2	+	1
Spleen			+	1
Cecal		1	+	1
Cecal tonsil	+	2		
Blood				
Bile/gall bladder	+	1	+	1
Stagnant water	+	1	+	1
Second follow-up visit for sample collection				
6 samples/category	Flock A		Flock C	
Samples	PCR: +/-	Number of + samples	PCR: +/-	Number of + samples
Feed				
Feces				
Liver				
Bile/gall bladder	+	4		
Ground soil				

SURVEY OF *EIMERIA* SPECIES AND ANTICOCCIDIAL RESISTANCE IN TURKEYS

H. Abdullah, A. Duff, K. Chasser, M. Trombetta, K. McGovern, and L. Bielke

Animal Sciences, College of Food Agriculture and Environmental Sciences, Ohio State University

SUMMARY

In poultry, coccidiosis is caused by species of *Eimeria*, a widespread protozoan parasite that causes gastrointestinal disease with consequences ranging from decreased growth performance during subclinical infections to high mortality in cases of infection with extremely pathogenic strains. Broilers represent the most economically important host, but *Eimeria* are pervasive across all poultry, including layers and turkeys. Estimates from 2016 data suggest that coccidiosis costs the US chicken industries $1.6 billion per year with an annual global cost of $14.4 billion (1). Inclusion of anticoccidial drugs in the feed and immunization with live oocysts are the primary means of prevention. An estimated 88% of US broilers raised on anticoccidial compounds, and though this has not been reported for turkeys, it is reasonable that, given limited vaccine options, a higher percentage are raised with anticoccidial medications (1,2).

Rotation and shuttle programs have become popular for limiting drug resistance but despite these efforts, multi-drug resistance is prevalent within flocks (Table 1) and was documented in the early 20th century, not long after anticoccidial medications were developed, and has continued despite efforts to alternate drugs in a logical pattern (3-8). Both types of program alternate medication, often based on mechanism of action, to delay the onset of resistance and extend the useful life of these products (9). The concept behind these alternating medications is that if resistance develops to one compound, then it will be lost during application of the next, but this philosophy remains unproven (2). Rotation programs involve providing a single anticoccidial medication for multiple cycles before rotating to a new medication and may include vaccination cycles. Typically, an entire complex uses the same medication for two to three months. Shuttle programs alternate anticoccidial medications more frequently with feed changes. Thus, birds are fed two or more medications within a flock when they change between starter, grower, and finisher feeds. There is no doubt that anticoccidial rotation and shuttle programs prolong the onset of resistance but given the prevalence of multi-drug resistant strains of *Eimeria* in today's poultry flocks, it could easily be argued that this strategy does not prevent resistance, and systematic testing to establish more effective rotation programs is necessary to the continued efficacy of anticoccidial compounds for the control of coccidiosis in poultry.

Extensive drug usage within intensive rearing systems is widely known to lead to resistance across all types of medications and concerns regarding multi-drug resistance in coccidia were recognized shortly after their development (10). It has also been expected that cross-resistance would occur, especially within class or mechanism of action such as monovalent ionophores. In fact, a 1975 research article reported multi-drug resistance to anticoccidials while mimicking a shuttle program for 40 passages and suggested that shuttle programs do not prevent resistance but can only delay it (11). Rathinam and Chapman (12) reported multi-drug resistance of turkey *Eimeria* field isolates and low incidence of sensitivity to amprolium and monensin. Thus, the pervasive existence of multi-drug resistant *Eimeria* in today's poultry industries is not unexpected, but it is approaching a critical threshold in which many producers may be left without medication options for prevention and treatment of coccidiosis.

In a review of anticoccidial compound resistance, Chapman previously suggested that sensitivity could be restored following passage of resistant lines via administration of an unrelated compound to birds (10). Sensitivity to salinomycin was restored in an experiment in which broilers infected with the resistant strain were fed a diet containing diclazuril or vaccinated in various combinations across four passages, suggesting that rotation programs, especially those that include vaccination, can have some success (13). However, this describes testing only one sensitivity profile and does not investigate other rotation programs for restoration of sensitivity. Since turkey producers have a longer growing cycle and limited medication options for control of coccidiosis, controlling resistance and restoration of sensitivity is more complicated than broiler flocks. Turning to

strategies employed by chicken breeder and layer arms of the poultry industry may prove useful, but limited vaccine coverage of species prevalent in commercial operations limits effectiveness of these options.

The Poultry Enteric Health Research Laboratory (PEHRL) recently established a program with turkey and chicken veterinarians to test fecal samples containing *Eimeria* for anticoccidial sensitivity (TACS) that has highlighted the prevalence of resistance in US turkey flocks (Figure 1). Table 1 summarizes sensitivity profiles across all flocks tested in the TACS program, and an astonishing 62% are multi-drug resistant while 26% are pan-resistant. All poultry operations producing birds under raised without antibiotics standards in the US also have limited anticoccidial choices because some drugs are classified as antibiotics. This decreases the amount of "down time" for any selected medication in rotation and shuttle programs and may promote resistance. While a vaccine is currently available to the turkey industry and can help manage anticoccidial sensitivity, this is only effective against the two species provided. Thus, turkey producers need to be aware of species affecting their flocks. A recent report on species detected among 33 turkey flocks in Canada showed an average of 2.8 species per flock with *E. gallopavonis* or *E. meleagridis* present in 48% [14]. Vaccinated flocks had fewer average species diversity, but at 2.4 species per flock, it can be assumed that vaccine containing two species does not offer full protection. This information is consistent with species detection in the TACS program at PEHRL (data not shown).

Adding a period of vaccination to a rotation program has been embraced by some integrators to re-establish drug-sensitive strains on farms (2,15). This strategy relies on re-seeding litter with drug-sensitive vaccine strains in place of drug-resistant strains as older oocysts age and die within litter during the vaccine cycle. However, recent studies have indicated that this strategy may not effectively alter drug sensitivity profiles within farms, where despite vaccinating with *Eimeria* sensitive to all medications, resistance to some was still detected after two cycles of vaccination (8). These variable results highlight remaining questions regarding how many consecutive grow-outs should be applied to overcome resistance to anticoccidials.

Anticoccidial sensitivity testing is recognized as a valuable tool by the scientific community for monitoring rotation and shuttle programs that can assist integrators in their *Eimeria* control programs (16). In 2020, PERHL established a TACS program for turkey integrators to screen farms and complexes to help them rotate medications likely to be effective against *Eimeria* established in their barns. The TACS service tests *Eimeria* samples against seven popular drugs for sensitivity and identifies species within samples via PCR. Identification provides information regarding the possibility that vaccination with the only commercially available option will help control coccidiosis since it contains only two of the six species known to infect turkeys. As of April 2021, nearly 150 samples have been received with 34 samples going through the entire TACS process. Nearly 100% of samples collected for testing contained *Eimeria*, which reveals the magnitude of the lack of coccidiosis control in turkeys.

Of the 34 farm samples submitted to TACS, 14% were pan-sensitive and 100% exhibited reduced sensitivity to at least one anticoccidial compound. If multi-drug resistance is classified as resistance to three or more anticoccidial compounds, 62% of samples are multi-drug resistant. Furthermore, 26% of farms exhibited either no sensitivity to all drugs or only one drug (Table 1).

In 2019, the Association of Veterinarians in Broiler Production reported to the USAHA (17) that coccidiosis is the disease of highest concern in broiler production. Alongside this, the National Turkey Federation cites coccidiosis as a top concern (#8) and the Association of Veterinarians in Egg Production reported coccidiosis as the first disease of concern in cage-free pullets, second highest disease of concern in caged pullets and the fourth highest of both caged and cage-free layers. These rankings arguably make coccidiosis and its control the highest disease priority of the US poultry industries. Taken into context with multi-drug resistance reports mentioned above, it becomes apparent that coccidiosis is a ticking time bomb that can devastate a major agriculture sector within years if effective control measures are not developed. While live oocyst vaccination is a strategy that can help, it is not a solution that works on its own and control of coccidiosis continues to largely depend on routine use of anticoccidial drugs (18). Thus, without introduction of new drugs to control coccidia and multi-drug resistance rising, integrators are facing fewer and fewer options to keep their flocks healthy. Turkey producers need to be pro-active in their approach to management of coccidiosis through judicious use of anticoccidial compounds, resistance and species monitoring, and application of vaccines where warranted.

REFERENCES

1. Blake DP, Knox J, Dehaeck B, Huntington B, Rathinam T, Ravipati V, et al. Re-calculating the cost of coccidiosis in chickens. Veterinary Research. 2020;51: 115. doi:10.1186/s13567-020-00837-2.

2. Chapman HD, Jeffers TK. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. International Journal for Parasitology: Drugs and Drug Resistance. 2014;4: 214–217. doi:10.1016/j.ijpddr.2014.10.002.

3. Cuckler AC, Malanga CM, Basso AJ, O'neill RC. Antiparasitic Activity of Substituted Carbanilide Complexes. Science. 1955;122: 244–245. doi:10.1126/science.122.3162.244-a.

4. Warren EW, Ball SJ, Mackenzie DR. The Incidence of Drug-Resistant Strains of Eimeria Species in Chickens in Great Britain, 1964/65. British Veterinary Journal. 1966;122: 534–543. doi:10.1016/S0007-1935(17)40256-9.

5. Hodgson JN, Ball SJ, Ryan KC, Warren EW. The Incidence of Drug Resistant Strains of Eimeria in Chickens in Great Britain, 1966. British Veterinary Journal. 1969;125: 31–35. doi:10.1016/S0007-1935(17)49161-.5

6. Martin AG, Danforth HD, Barta JR, Fernando MA. Analysis of immunological cross-protection and sensitivities to anticoccidial drugs among five geographical and temporal strains of eimeria maxima. International Journal for Parasitology. 1997;27: 527–533. doi:10.1016/S0020-7519(97)00027-1.

7. Bafundo KW, Cervantes HM, Mathis GF. Sensitivity of Eimeria Field Isolates in the United States: Responses of Nicarbazin-Containing Anticoccidials. Poultry Science. 2008;87: 1760–1767. doi:10.3382/ps.2008-00129.

8. Snyder RP, Guerin MT, Hargis BM, Kruth PS, Page G, Rejman E, et al. Restoration of anticoccidial sensitivity to a commercial broiler chicken facility in Canada. Poultry Science. 2021;100: 663–674. doi:10.1016/j.psj.2020.10.042.

9. Chapman HD. Use of Anticoccidial Drugs in Broiler Chickens in the USA: Analysis for the Years 1995 to 1999. Poultry Science. 2001;80: 572–580. doi:10.1093/ps/80.5.572.

10. Chapman HD. Biochemical, genetic and applied aspects of drug resistance in Eimeria parasites of the fowl. Avian Pathology. 1997;26: 221–244. doi:10.1080/03079459708419208.

11. McLoughlin DK, Chute MB. Sequential Use of Coccidiostats: Effect on Development by Eimeria tenella of Resistance to Amprolium, Nicarbazin, Unistat, and Zoalene. Avian Diseases. 1975;19: 424–428. doi:10.2307/1589067.

12. Rathinam T, Chapman HD. Sensitivity of Isolates of Eimeria from Turkey Flocks to the Anticoccidial Drugs Amprolium, Clopidol, Diclazuril, and Monensin. Avian Diseases. 2009;53: 405–408. doi:10.1637/8679-030509-Reg.1.

13. Chapman HD, Jeffers TK. Restoration of sensitivity to salinomycin in Eimeria following 5 flocks of broiler chickens reared in floor-pens using drug programs and vaccination to control coccidiosis. Poultry Science. 2015;94: 943–946. doi:10.3382/ps/pev077.

14. Imai R, Barta J. Diversity and Cross-Immunity of Eimeria species infecting Turkeys in Commercial Flocks in Canada. 2018. Available: http://hdl.handle.net/10214/13051.

15. Mathis GF, Broussard C. Increased Level of Eimeria Sensitivity to Diclazuril After Using a Live Coccidial Vaccine. Avian Diseases. 2006;50: 321–324. doi:10.1637/7455-101305R1.1.

16. Peek HW, Landman WJM. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. Veterinary Quarterly. 2011;31: 143–161. doi:10.1080/01652176.2011.605247.

17. USAHA. Report of the USAHA Committee on Poultry and Other Avian Species. In: United States Animal Health Association [Internet]. 2019 [cited 20 Apr 2021]. Available: https://www.usaha.org/transmissible-diseases-of-poultry-avian-species.

18. Williams RB. Anticoccidial vaccines for broiler chickens: Pathways to success. Avian Pathology. 2002;31: 317–353. doi:10.1080/03079450220148988.

Anticoccidial sensitivity classification	% Flocks Tested
Pan-sensitive	14%
Pan-resistant	26%
Multi-drug resistant	62%
Sensitive or reduced sensitive ≥ 4	38%

Table 1. Anticoccidial sensitivity classification of farm samples tested by PERHL. Pan-sensitive = sensitive or reduced sensitivity to all drugs; Pan-resistant = sensitive to ≤ 1 drug; multi-drug resistant = resistant to ≥ 3 drugs

Figure 1. Representative results of test for anticoccidial sensitivity (TACS) of fecal samples from turkey integrators. PERHL has completed nearly 40 TACS samples to date.

Farm	Farm 1	Farm 2	Farm 3	Farm 4
Flock Age	36 days	36 days	14 days	5 weeks
Amprolium	SENSITIVE	RESISTANT	RESISTANT	REDUCED SENSITIVITY
Lasalocid	SENSITIVE	SENSITIVE	RESISTANT	RESISTANT
Monensin	SENSITIVE	REDUCED SENSITIVITY	RESISTANT	-REDUCED SENSITIVITY
Clopidol	SENSITIVE	SENSITIVE	SENSITIVE	SENSITIVE
Halofuginone	REDUCED SENSITIVITY	SENSITIVE	REDUCED SENSITIVITY	RESISTANT
Zoalene	RESISTANT	SENSITIVE	RESISTANT	REDUCED SENSITIVITY

FIELD EXPERIENCE MANAGING A PRECOCIOUS COCCIDIOSIS VACCINE IN BROILER BREEDERS UNDER FIELD CONDITIONS IN ZIMBABWE, SOUTH AFRICA

J.J. Bruzual[A] and C. Archenoul[B]

[A]Aviagen, Inc. Huntsville, Alabama
[C]Hukuru Chicks. Ruwa, Zimbabwe

SUMMARY

Coccidiosis is a highly prevalent protozoan disease affecting chickens worldwide. Some companies implement the use of anticoccidial drugs to manage coccidiosis in broiler breeders, but many times this approach does not provide the desired long-term immunity, affecting livability and production. To address these challenges, three consecutive broiler breeder flocks (Ross 344 male x Ross 308 females) were vaccinated with a commercial precocious coccidiosis vaccine at the hatchery. Following vaccination, the birds were moved to the farm and raised under proper brooding conditions. Furthermore, appropriate density of birds and feeder space were ensured based on bird age, trying to reach 25-35% litter humidity to guarantee sporulation and recycling of oocysts, needed for optimal immunity against coccidiosis.

Results in the grow out phase showed improved flock uniformity, a decrease in mortality and costs associated with coccidiosis treatments.

In conclusion, a precocious coccidiosis vaccine can be used to effectively immunize broiler breeders, improving livability and associated costs.

TRADITIONAL SURVEY-BASED METHODS FOR MAPPING BACKYARD AND GAME FOWL POULTRY MOVEMENT IN CALIFORNIA

M. Cadena[A] and M. Pitesky [B]

[A]UC Davis, Animal Biology
[B]UC Davis, School of Veterinary Medicine, Population Health and Reproduction- Cooperative Extension

SUMMARY

The 2018-2020 outbreak of virulent Newcastle disease (vND) in Southern California is the third major outbreak of this foreign animal disease in California within a 50-year time span. Outbreaks have been primarily driven by unvaccinated backyard chickens (BYC) and game fowl (GF) (i.e. fighting roosters) transmitting the virus to commercial operations in the region. In an attempt to evaluate the risk of disease transmission in BYC and GF, an online survey linked to network analysis was administered. Specifically, poultry owners were asked about their coop, husbandry/biosecurity practices in addition to trading practices for the network analysis. These data ultimately are essential for improved disease modeling and hence preparation and response for future vND outbreaks. Of the total 104 participants, 99 identified as backyard chicken owners, 0 identified as game fowl owners and five identified as both backyard and game fowl owners.

INTRODUCTION

The 2018-2020 outbreak of virulent Newcastle disease (vND) in Southern California is the third major outbreak of this foreign animal disease in California within a 50-year time span, the first occurring from 1971-1974 and the second from 2002-2003 (1). The outbreaks were primarily driven by unvaccinated backyard chickens (BYC) and game fowl (GF) transmitting the virus to commercial operations in the region (1). In 2002 alone, the spill over into commercial poultry resulted in $121 million USD in trade losses (3). Moreover, it is estimated that 3.2 million birds were depopulated resulting in losses of $160 million USD to eradicate the disease (3).

The emergence of vND remains an ongoing threat to the US with vND considered endemic in Mexico, Latin America, and other parts of the world. Furthermore, survey studies indicate that BYP ownership is increasing in California and nationally (6). This is concerning as previous studies indicate that vND vaccine compliance in BYP is extremely poor: approximately 2.8% of all flocks surveyed (2).
The devastating and costly outbreaks that have primarily affected BYP and game fowl communities of diverse ethnicities and cultures, demonstrates the need to address social and cultural factors in disease modeling and eradication programs for vND. While there have been studies that surveyed BYP and GF in the US, they were performed over two decade ago (6–8) and are limited in scope. Specific limitations include lack of granular location and movement data. For instance, in a study by USDA (2004), participants that introduced new birds to their flock were asked if the source location of new birds were from: within same county, outside county but within state, outside state but within the United States, and so on (7). In other words, connectivity and distance traveled between regions is unknown and understudied. Data that is paramount for disease modeling and targeted outreach efforts.

We propose to utilize network analysis, a scientific and quantitative way to study and visualize relationships (4), to quantify and visualize live bird movement patterns between counties in California. Network analysis coupled with Geographic Information Systems (GIS) could help us identify counties that import and export the most BYC and GF. In this study, an online, GIS-based survey and network analysis were used to map BYP and GF movement and husbandry practices. The study aimed to better understand the spread of vND in California.

MATERIALS AND METHODS

Study sample. The target populations for the survey were backyard chicken owners and game fowl breeders from California. For this survey, backyard chickens were defined as chickens primarily kept for eggs and/or meat at residences. Game fowl were

defined as breeds of chickens, such as Kelso, Hatch, Claret, and Roundhead, intended primarily for exhibition/competition and bred for beauty, strength, health, vitality, and longevity (2).

Survey design and data collection. An online survey was created in English using the survey instrument Qualtrics XM. The survey consisted of 3 main sections:

1. Information about the BYP owner
2. Biosecurity practices
3. Live bird movement
    - Purchasing
    - Trading
    - Selling
    - General movement to areas where other birds are present (e.g. fairs, shows, etc.)

For questions that asked about location, survey participants were asked to provide county-level information as address information is more sensitive. The Institutional Review Board at the University of California, Davis approved the survey (IRB ID 1715861).

Recruitment of BYP owners. The survey was hosted on the University of California Cooperative Extension (UCCE) Poultry website and shared with farm advisors and media coordinators from UC Davis, UC Agriculture and Natural Resources (UC ANR) and the California Department of Food and Agriculture (CDFA). Additionally, since there are various backyard chickens and game fowl groups on Facebook, a Facebook page was created for the survey and promoted using Facebook's Ad Center. The survey was open from August to October 2021.

Network analysis. For this paper, only results from the live bird movement section of the survey will be reported. Relational matrices were created and analyzed using Microsoft Excel. In-degree and out-degree centralities were calculated for each county in the networks. In this study, in-degree refers to the number of times a survey respondent indicated they moved birds into the county in the previous 12 months. In contrast, out-degree refers to the number of times a survey respondent indicated they moved birds out of the county in the previous 12 months. Attribute tables were also built in Microsoft Excel. Counties were geocoded using the geocoder in ArcGIS 10.7.1. Then the networks were visualized using R 4.1.2 and R Studio 2021.09.2 with the statnet, network and maps packages. Nodes represent counties and ties represent live bird movement. Commercial poultry data from 2016 was also mapped to better understand the risk to commercial poultry, as well.

RESULTS

Participants. In total, there were 104 survey participants. Of the 104 survey participants, 99 indicated they only had BYC. The remaining five owners indicated they had both BYC and GF (BYC-GF). No survey participants indicated they only had game fowl. Since the survey was distributed online, the total number of survey invitations is not known. Therefore, response rates are not reported here.

Network analysis. Two movement networks were built, one for BYC owners and one for BYC-GF owners. As seen in Table 1, in the BYC network, 23 counties out 58 California counties (40%) were represented. In the BYC-GF network, 12 counties were represented (21%) (Table 1). In the BYC network, the county with the highest in-degree centrality of eight was Nevada county. In the BYC-GF network, both Nevada and Glenn and county had the highest in-degree centrality of eight, as well. When looking at which counties had the most respondents indicate they moved birds out of the county in the previous 12 months, Nevada county was the county with the highest out-degree in the BYC network. In the BYC-GF network, Glenn county had the highest out-degree centrality of 10.

As seen in Figure 1A, there was connectivity between Northern and Southern California and some proximity to commercial farms (purple nodes) in the BYC network. Additionally, some of the Northern counties had a large in-degree centrality compared to the counties in Southern California (Figure 1A). For example, Nevada, Yolo, Sacramento and Sonoma county had an in-degree centrality of 8, 5, 4 and 4, respectively (Table 1). In the BYC-GF network, most of the connectivity occurred in Northern California with some proximity to commercial farms (Figure 1B) similar to the BYC network (Figure 1A). Interestingly, Nevada county was also the county with the highest in-degree centrality (centrality = 8) along with Glenn county (Table 1).

In terms of out-degree centrality, the BYC network had counties in both Northern and Southern California with high centralities (Figure 2B). Specifically, Nevada, Sacramento, San Diego, Sonoma, Ventura and Yolo county had out-degree centralities of 6, 4, 4, 4, 4, and 4 respectively (Table 1). In the BYC-GF network, Glenn County had the highest out-degree centrality (centrality = 10) in addition to a high in-degree centrality (centrality= 8) as mentioned earlier (Table 1). All the other counties had an out-degree centrality of four or less (Table 1).

DISCUSSION

The survey results provided some important insights such as the connectivity between Southern and Northern California and with counties affected by vND in the BYC network (Figure 1A and Figure 2A). However, the survey also had significant limitations and biases. For example, out of the estimated 50,000 to 60,000 GF owners in California (9), only five GF owners that also owned BYC participated and only one was from a county affected by vND (Figure 1B). Overall, there was poor participation from the areas historically affected by vND in Southern California. A likely cause for the lack of representation from this region was that the survey was only available online and only in English.

Although conducting another survey that heavily targets GF owners and the areas historically affected by vND could be done, the sensitive nature of owning breeds historically bred for fighting may still make it difficult to obtain location and movement information. In addition, it may be difficult to capture the seasonality of movement patterns (e.g. less movement during the molting season) with just one or a few surveys. While a larger boots-on-the-ground effort employing multiple language in-person surveys could be attempted in these vND-affected areas, it is economically and logistically prohibitive to perform in states as large and diverse as California. Additionally, there are limited extension resources focused on poultry in the state, with only two University of California Cooperative Extension (CE) poultry specialists and no poultry farm advisors.

One potential solution or way to augment traditional survey methods is to utilize computational social science techniques to obtain dynamic data. Social web data (e.g. birds for sale posts) could potentially be used as a proxy or as a supplement to traditional survey data (5), and subsequently used to inform extension and disease eradication efforts. The use of these non-traditional methods to augment traditional survey methods will be explored in future studies.

ACKNOWLEDGEMENTS

Funding was provided by the California Department of Food and Agriculture. Additionally, we would like to thank the farm advisors and media coordinators that helped us distribute the survey. Lastly, we thank the bird owners that participated in this study.

REFERENCES

1. Dimitrov, K. M., H. L. Ferreira, M. J. Pantin-Jackwood, T. L. Taylor, I. V. Goraichuk, B. M. Crossley, M. L. Killian, N. H. Bergeson, M. K. Torchetti, C. L. Afonso, and D. L. Suarez. Pathogenicity and transmission of virulent Newcastle disease virus from the 2018–2019 California outbreak and related viruses in young and adult chickens. Virology 531: 203–218. 2019.

2. Garber, L., G. Hill, J. Rodriguez, G. Gregory, and L. Voelker. Non-commercial poultry industries: Surveys of backyard and gamefowl breeder flocks in the United States. Prev. Vet. Med. 80: 120–128. 2007.

3. Hietala, S., H. Kinde, B. M. Crossley, and A. Ardans. Exotic Newcastle disease in California: laboratory response to an animal health emergency. Can. Vet. J. Rev. Vét. Can. 45: 1022. 2004.

4. Knoke, D., and S. Yang. Social Network Analysis 2nd ed. Thousand Oaks, California. 2022.

5. Pastor-Satorras, R., C. Castellano, P. Van Mieghem, and A. Vespignani. Epidemic processes in complex networks. Rev Mod Phys 87: 925–979. 2015.

6. USDA. Part I: Reference of Health and Management of Backyard/Small Production Flocks in the United States, 2004. 2004.

7. USDA. Part II: Reference of Health and Management of Gamefowl Breeder Flocks in the United States, 2004. 2004.

8. USDA. Urban Chicken Ownership in Four U.S. Cities. 2013.

9. USDA. Foreign Animal Disease Preparedness & Response Plan. 2013.

Table 1. Summary of in-degree and out-degree centrality for counties in the BYC and BYC-GF networks. The counties with the highest in-degree and out-degree centrality are highlighted in grey.

	BYC		BYC-GF	
County	In-degree centrality	Out-degree centrality	In-degree centrality	Out-degree centrality
Alameda	3	2		
Butte			4	4
Calaveras	0	1		
Colusa			0	2
Contra Costa	0	1		
El Dorado	0	1		
Fresno			2	2
Glenn			8	10
Humboldt	1	1		
Los Angeles	4	2	4	4
Marin	2	2		
Monterey	3	3		
Nevada	8	6	8	0
Placer	2	3	2	0
Riverside	2	1		
Sacramento	4	4		
San Diego	2	4		
San Joaquin	2	0	0	2
Santa Barbara	0	1		
Santa Cruz	1	1		
Shasta			2	0
Sierra			0	2
Solano	1	0		
Sonoma	4	4		
Stanislaus	0	1		
Sutter	1	2	0	2
Tehama			0	2
Tulare	2	1		
Ventura	2	4		
Yolo	5	4		

[A] Some counties do not have data because they were part of just one network. For example, no backyard chicken owners indicated they sold, traded or bought chickens from Butte county but some game fowl owners did.

Figure 1. Movement network with node size reflecting in-degree centrality (ie. the higher the centrality, the larger the node) for BYC (A) and BYC-GF (B). Nodes represent counties and links represent bird movement. Nodes from counties that were affected by vND in 2018 are colored red, the rest are red. Purple nodes represent commercial poultry.

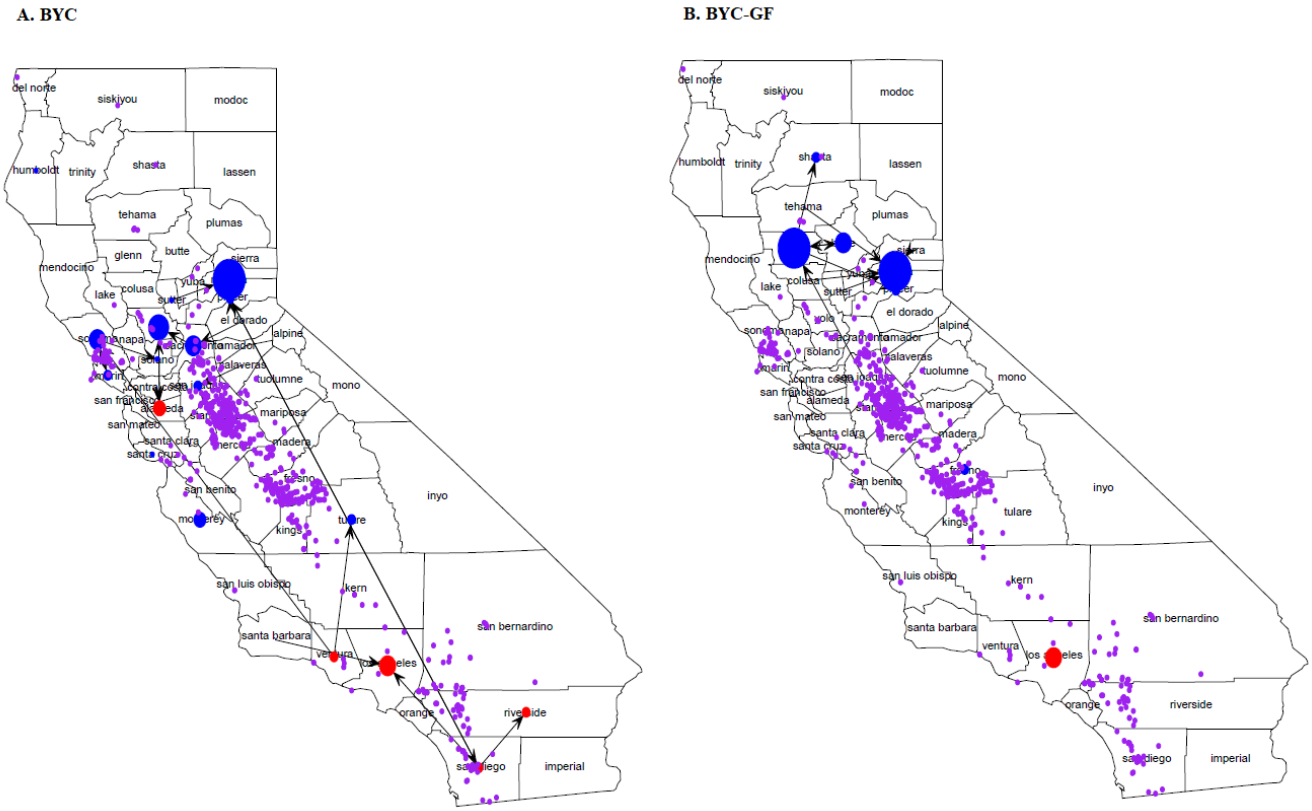
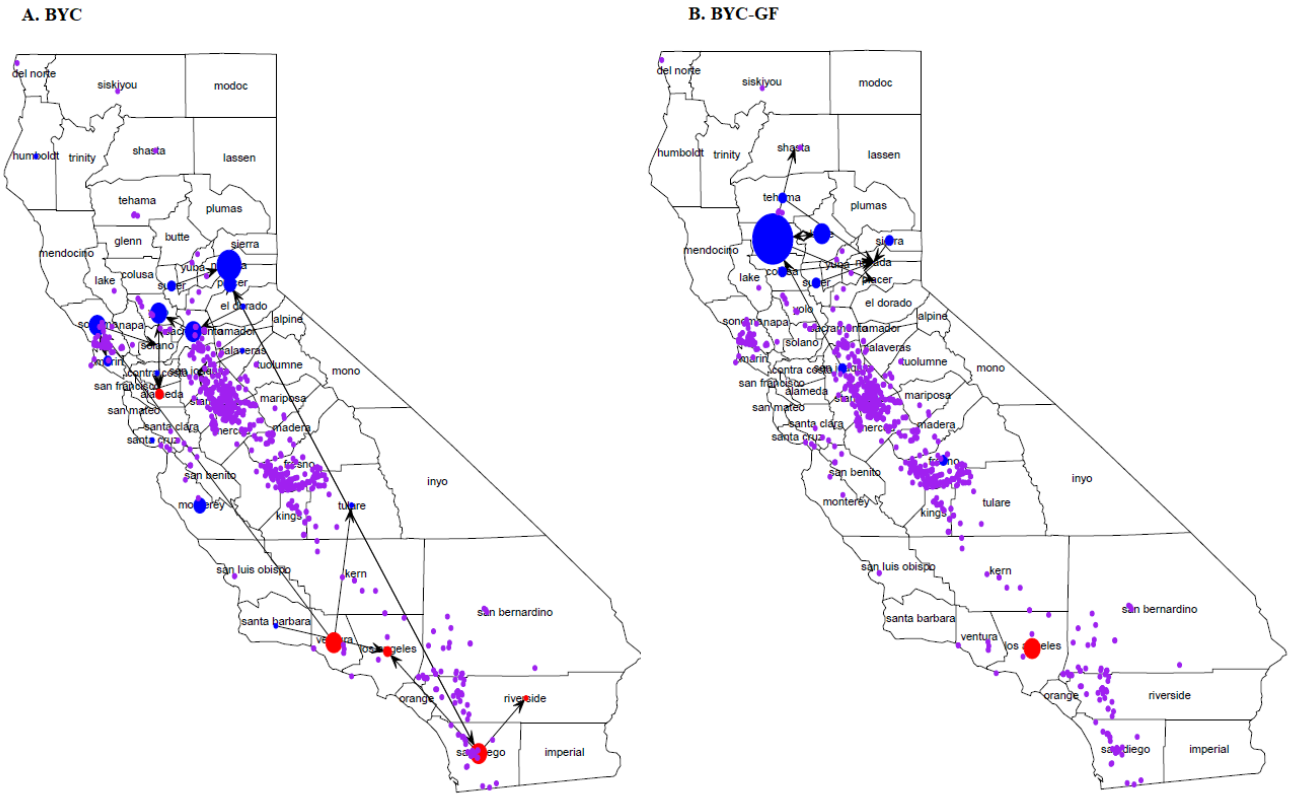


Figure 2. Movement network with node size reflecting out-degree centrality (ie. the higher the centrality, the larger the node) for BYC (A) and BYC-GF (B). Nodes represent counties and links represent bird movement. Nodes from counties that were affected by vND in 2018 are colored red, the rest are red. Purple nodes represent commercial poultry.



EPIDEMIOLOGICAL TOOLS FOR INFORMING DISEASE RISK: THE EXAMPLE OF AVIAN INFECTIOUS BRONCHITIS

M. Campler[A], Ting-Yu Cheng[A], C. Hofacre[B], and A. Arruda[A]

[A]The Ohio State University, Department of Veterinary Preventative Medicine, Columbus, OH 43210
[B]Southern Poultry Research Group, Inc., Watkinsville, GA, 30607

INTRODUCTION

The highly contagious avian infections bronchitis virus (IBV) remains a global poultry production issue. Despite increased focus on biosecurity, vaccination strategies and enhanced screening methodology to quickly identifying outbreaks, the high mutation and recombination rate of IBV still enables the virus to be detrimental to poultry welfare and production (1). As no commercial vaccine for IBV is widely available to date, and due to the relatively short-term protection of inactivated vaccines often used on-farm, antibody titer of flocks are routinely used to determine vaccine potency, immune uniformity and potential wild-virus exposure (2). Although antibody titers allow for the detection of previous infection of the flock, less is known about the impact of the environment surrounding the farm and its potential impact on local flock titer levels and viral exposure. Previous studies have discussed the possibility of natural vegetation acting as particle buffers both in farm and urban regions (3, 4) and have specifically been discussed for respiratory virus transmission between commercial poultry farms (5). However, there is a large knowledge gap regarding the impact of other environmental factors in the farm landscape. Currently, only farm density has been linked to increased risk of IBV spread (6).

Thus, the objective of this study was to use a combination of geospatial software, machine learning-based variable selection algorithms, poultry health data, and mixed effect modeling to determine factors associated with elevated IBV antibody titers in commercial broiler farms.

MATERIALS AND METHODS

Health data including IBV antibody titer levels from 1,111 sampling events in 166 commercial broiler chicken farms located in Midwestern United States was obtained retrospectively for a period of approximately five years (fall 2016 to spring 2021). The date of sampling, age of sampled birds (in weeks), mean IBV titer level and farm identification along with latitude and longitude were also provided. Elevated IBV antibody levels (yes/no) for each sampling event was determined using an ELISA cut-off titer level of 396 (7).

Geospatial data for the region was obtained using publicly available data sets for which sources included the United States Department of Transportation (USDOT), the United States Geological Survey (USGS), and the United States Department of Agriculture (USDA, APHIS). The obtained geospatial data included topography, land cover, waterways, slaughter plant locations, and road networks. In addition, the location of other poultry farms in the region, including their type of operation, size and housed poultry commodity (turkey, broiler, layer and pullet) was obtained and projected using a EPSG:4326-layer projection over the official Google Satellite Base map for QGIS 3.18.1 (8,9). Based on the known location of study farms in relation other poultry farms in the region, farm density for the total number of farms/km^2 and per 2-km-radius for each specific farm type and commodity was calculated. In addition, the proximity to each farm type and commodity as well for each geospatial feature of interest was calculated for each study farm. Land cover type, altitude and terrain slope were extracted for each study farm in QGIS.

Variable selection was processed first using a random forest machine learning Boruta algorithm in R. 4.0.4 (10); which followed criteria of only choosing variables with a higher estimated compared to the randomized subset of the original data (shadow features). Second, statistical modeling proceeded by checking pre-selected variables for linearity and checking correlation between variables before building a final multivariate mixed logistic regression model using backward elimination processes. The final model included year, age, and distance to commercial layer farms. The lack of independence of sampling events due to the repeated farm sampling was considered using a random effect.

RESULTS AND DISCUSSION

The majority land cover distribution across the 166 study broiler farms was pasture/hay (60%) followed by developed areas (20%), cultivated crops (10%), forest (8%), and others (2%), which included herbal grasslands, scrubland, or wetland. The average distance to any poultry farm was 0.67 km ± 0.67 SD. The average distance to each poultry commodity was 0.74 ± 0.59, 0.67 ± 0.63, 1.35 ± 0.97 and 1.97 ± 1.1, km ± SD, for broiler, layer, pullet and turkey farms, respectively. The average farm density for each poultry commodity was 10.8 ± 3.83, 10.1 ± 3.25, 2.28 ± 0.5, and 0.7 ± 0.31 farms/km^2, ± SD, for broiler, layer, pullet or turkey farms, respectively.

The lack of impact of the geospatial variables on the risk of elevated IBV antibody titer-levels in this study was surprising. Farm density has previously been suggested to IBV spread but was not observed (6). However, the multivariable mixed logistic regression model showed that the risk of increased IBV titer levels fluctuated heavily between years compared to our reference year 2017 (Table 1). However, no effect of seasonality was observed nor retained in the final model. Birds older than 4 weeks of age tended to have a 37% increase in IBV antibody titer levels compared to younger birds, possibly indicating higher odds of viral exposure later in life. Farms located more than 3.2 km and 6.4 km from a commercial layer farm had a 1.5 to 2-fold increase in IBV anti-body titer levels. This counter intuitive finding may however be caused by factors not measured or captured by our current modeling approach, such as the health status for the flocks on the surrounding farms. It could also be explained by the fact that biosecurity might be enhanced if surrounding farms are perceived as a risk for producers.

In conclusion, this study briefly exemplified the approach of using publicly available data in combination with geospatial mapping, variable selection using machine learning, and animal health data, which is commonly available from production systems. It also highlights the need of a centralized system for health data mapping in the poultry industry which would be valuable for further investigation into IBV and other poultry diseases through comprehensive risk factor analyses, which could lead to improvement on disease prevention and control at a broader level.

(A full version of this manuscript will be published in *Avian Diseases*.)

REFERENCES

1. Yilmaz H, Altan E, Cizmecigil UY, Gurel A, Ozturk GY, Bamac OE, Aydin O, Britton P, Monne I, Cetinkaya B, Morgan, KL, Faburay, B, Richt JA, Turan N. Phylogeny and S1 gene variation of infectious bronchitis virus detected in broilers and layers in Turkey. Avian Dis, 60(3):596-602, 2016.
2. De Herdt P, Ducatelle AR, Uyttebroek AE, Sneep A, Torbeyns R. Infectious bronchitis serology in broilers and broiler breeders: Correlations between antibody titers and performance in vaccinated flocks. Avian Dis, 45: 612–619
3. Adrizal A, Patterson PH, Hulet RM, Bates RM, Myers CA, Martin GP, Shockey RL, van der Grinten M, Anderson DA, Thompson JR. Vegetative buffers for fan emissions from poultry farms: 2. ammonia, dust and foliar nitrogen. J Environ Sci Health B, 43(1):96:103. 2008.
4. Janhäll S. Review on urban vegetation and particle air pollution – deposition and dispersion. Atmospheric Environment 2015; 105:130-137. 2015.
5. Burley HK, Adrizal A, Patterson PH, Hulet RM, Bates RM, Martin GP, Myers CAB, Atkins HM. The potential of vegetative buffers to reduce dust and respiratory virus transmission from commercial poultry farms. J Appl Poultry Res, 20(2):210-222. 2011.
6. Franzo, G., Tucciarone, C.M., Moreno, A., Legnardi, M., Massi, P., Tosi, G., Trogu, T., Ceruti, R., Pesente, P., Ortali, G., Gavazzi, L., Cecchinato, M. Phylodynamic analysis and evaluation of the balance between anthropic and environmental factors affecting IBV spreading among Italian poultry farms. Sci Rep, 10: 7289. 2001.
7. Manswr B, Ball C, Forrester J, Chantrey J, Ganapathy K. Host immune response to infectious bronchitis virus Q1 in two commercial broiler chicken lines. Res Vet Sci, 136:587-594. 2021.
8. Patyk, K. A., McCool-Eye, M. J., South, D. D., Burdett, C. L., Maroney, S. A., Fox, A., Kuiper, G., & Magzamen, S. (2020). Modelling the domestic poultry population in the United States: A novel approach leveraging remote sensing and synthetic data methods. *Geospatial Health*, *15*(2). h
9. QGIS Development Team, 2021. QGIS Geographic Information System 3.18.1. Open-Source Geospatial Foundation Project. http://qgis.osgeo.org. Downloaded Mar 2021.
10. R Core Team, 2021. R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing: Vienna, Austria.

Table 1. Multivariable logistic regression analysis on the odds of higher IBV antibody titers in broiler chickens.

Variable		Odds Ratio	Std. Error	95% CI		P-Value
Year	2017[1]	Reference				
	2016[2]	5.65	2.22	2.61	12.2	<.001
	2018	1.67	0.43	1.0	2.80	.05
	2019	1.17	0.32	0.69	1.99	.56
	2020	4.22	1.07	2.57	6.94	<.001
	2021[2]	2.01	0.73	0.99	4.09	<.001
Age (week)	<4	Reference				
	>4	1.37	0.72	0.97	4.09	.05
Distance commercial layer farm (km)	<3.2	Reference				
	3.2-6.4	1.54	0.33	1.01	2.33	.04
	>6.4	1.99	0.44	1.29	3.01	.002

[1]Reference was chosen as it was the first year with a complete IBV antibody titer sampling data set for all seasons.
[2]Only sampling events from the fall and winter months were obtained.

INFECTIOUS BURSAL DISEASE VACCINE TYPE IMPACTS RESPONSE TO INFECTIOUS BRONCHITIS VACCINATION AND BURSAL INTEGRITY

N. Cariou[A] and E. Myers[A]

[A]MSD Santé Animale, 7, rue Olivier de Serres, 49070 Beaucouzé, France

SUMMARY

The impact of different infectious bursal disease (IBD) vaccines on bursal integrity and ELISA titers to infectious bronchitis (IB) were evaluated in day-old JA957 broilers in a research station. Birds were either unvaccinated for IBD (control) or subcutaneously vaccinated with either an immune complex (IC) IBD vaccine or a herpesvirus of turkeys dual-construct (HVT-DC) vaccine expressing the VP2 capsid protein of IBD virus and the F protein of Newcastle disease virus. At 15 days of age, the 3 groups received live IB vaccine via eye drop. Bursa were collected for bursa/bodyweight (B/BW) ratio and histopathology. IB serology was conducted, and mortality was recorded. At 49 days, in comparison to IC vaccinates, HVT-DC vaccinates had significantly lower mortality (p_{khi^2}=0,06), significantly higher IB ELISA titers (p=0,011), significantly higher B/BW ratio (p<0,001) and a significantly lower lymphoid depletion score (p<0,001).

INTRODUCTION

Infectious bursal disease (IBD) and infectious bronchitis (IB) are the two most common viral diseases of chickens in Europe, with immunosuppression and degraded technical performance being the most common form of IBD, while IB results in respiratory and renal disorders in broilers. Both viruses expose chickens to bacterial superinfections and the need for antibiotic therapy. Prevention is based on biosecurity and vaccination. Hatchery vaccination against IBD is being developed, based on two different technologies: immune complex (IC) or herpesvirus of turkeys (HVT) construct.

The aim of this study is to measure the possible consequences of an IBD vaccination at the hatchery on bursal integrity and the ELISA IB titers following IB vaccination uptake in commercial broilers.

MATERIALS AND METHODS

Birds. Day old JA957 broilers were sexed and randomly distributed among different repeats. Three hundred sixty chicks in the control group, group 1, distributed in 20 replicates (10 males and 10 females) of 18 birds, that were not vaccinated for IBD. For each treatment group, groups 2 and 3, 216 chicks were distributed in 12 replicates (6 males and 6 females) of 18 birds. To avoid cross-contamination between pens, following precautions were applied: animal care without going into pen, a plastic cover added on the low part of the sides of the pens, wooden partition (0.80 m high) was placed between group 3 and the 2 other groups with a footbath to use before moving from one area to another.

Vaccines. Day-old chicks in group 2 were vaccinated subcutaneously with herpesvirus of turkeys dual-construct (HVT-DC) vaccine expressing the VP2 capsid protein of IBD virus and the F protein of Newcastle disease virus. Day-old chicks in group 3 were subcutaneously vaccinated with an immune complex (IC) IBD vaccine, strain SYZA26.
At 15 days of age, all three groups received live IB vaccine via eye drop, containing strains 4-91 and Massachusetts.

Data collected. Clinical inspection and mortality was recorded daily. Twenty Blood samples were collected at day 0, maternal antibodies were measured by BioChek ELISA IBD kit, following the manufacturer's instructions. For all 3 groups 20 blood samples were collected at day 28, 35, 42, and 49 for BioChek ELISA IB kit, following the manufacturer's instructions.

In all 3 groups, ten tracheal swabs were collected at day 18 and ten cloacal swabs were collected at day 28, 35, and 49. All the samples received were tested with a real time RT-PCR test targeting a conserved region of the coronavirus genome. The partial sequencing of the S1 gene was further performed in all positive samples using the SANGER method. With the aim to classify the strains, the corresponding amino

acid sequences obtained were aligned and compared with the sequences of the common commercial IB vaccines used in the field.

Necropsies were performed on 5 broilers per group at day 15, 28, 35, and 4 broilers per group at day 49. Bursa weight/body weight (B/BW) ratio were calculated according to the following formula: [bursa weight (g)/body weight (g)] x100.

Four bursa per group at 15, 28, 35, and 49 days were collected, then split for histopathology and next generation sequencing (NGS) with the Viral Flex-Seq® (VFS) IBDv Field Virus assay: a multiplex PCR targets the hypervariable region of VP2. PCR products are sequenced using Illumina sequencing instruments. VP2 consensus sequences are aligned against a set of reference sequences representing distinct viral genogroups. The histopathologists did two evaluations of lymphoid depletion, the mean of the two was the final lymphoid depletion score.

Statistical analysis. Alpha risk is 5%. SAS® software, version 9.4 was used to run statistical tests. For quantitative trait, mortality, proportions were compared using X^2 test. For qualitative traits**,** IB titers, B/BW ratio, and lymphoid depletion score, fixed effects were tested using variance analysis.

RESULTS

Geometric mean for IBD titers at day old was 7024, higher than 2,500 with BioChek ELISA, in conformity with the IC vaccine's SPCs.

As expected, the dominant 4-91 vaccine strain was recovered by PCR on the 3 groups from day 18 tracheal swabs, mean CT= 19.5, and day 28, 35, and 49 cloacal swabs, with mean CT of 24.4.

No clinical signs were observed during the trial and only a few birds died during the trial. Mortality rate was higher in IC group (2.3%) than in HVT-DC group (0.9 %). In control group mortality rate was 0.3%. $pKhi^2 = 0.06$, the differences are significant for α-risk = 0.10.

There was a significant treatment effect on 42 and 49-day-old IB titers with more antibody for HVT-DC group (Graph 1).

42-day-old IB titers: HVT-DC group (2205) > Control group (2111) > IC group (1530). p = 0.055, significantly different for α-risk = 0.10 (Graph 2).

49-day-old IB titers: HVT-DC group (2682) > IC group (1936) > control group (1389) (p = 0.011) (Graph 3).

There is a significant treatment effect on 15 and 49-day-old B/BW ratio (Graph 4).

15-day-old B/BW ratio is lower in IC group (0,20%) than in HVT-DC and control group (0,25%). (p = 0.020)

49-day-old B/BW ratio is higher in HVT-DC group (0,16%) than in control group (0,05%) and IC group (0,04%). ($p < 0.001$) (Graph 5).

There is a significant treatment effect on 49-day-old lymphoid depletion score (Graph 6): 49-day-old score is higher in control group (88.5%) and IC group (74.5%) than in HVT-DC group (4.1%) ($p<0.001$) (Graph 7, Photo 1, and Photo 2).

DISCUSSION AND CONCLUSION

These results could be compared with those obtained by Lupini (2020) on SPF chickens, comparing the HVT-DC vaccine to an IC vaccine (based on strain Winterfield 2512), showing significantly higher IB titers in the HVT-DC vs IC group from 14days post-vaccination, an increase of CD8+ and a reduction of the CD4+/CD8+ ratio in the spleen, trachea, and harderian gland. Bursal atrophy from 14 days post IBD vaccination, measured by a lower B/BW ratio, was significantly more severe on IC vaccinates. In our study, in the presence of maternal antibodies delaying multiplication of IC vaccine strain, the ELISA IB titers are significantly higher in HVT-DC group from 35 days post vaccination. B/BW ratio are significantly higher at 15 days and 49 days in HVT-DC vaccinates vs IC vaccinates. Moreover, we confirmed the bursa integrity post HVT-DC vaccination with lymphoid depletion score, which is significantly lower at 49 days in HVT-DC group vs IC vaccinates, meaning no negative impact on immune response for HVT-DC vaccinates.

Following monitoring of IC vaccine replication by NGS, the IC vaccine strain started to be found in 2/4 chickens of the IC group at 35 days. At 49 days, it was found in 4/4 chickens of the IC group and in the 4 chickens of the control group, despite the precautions put in place to avoid the spread of the vaccine. It was not found in the HVT-DC group. In the control group, the IB titers, the B/BW ratio, and the lymphoid depletion were very close to those of the HVT- DC group until 35 days, but at 49 days the B/BW ratio decreased abruptly while lymphoid depletion increased, to approach those of the IC group. The IB titers of the control group, close to those of the HVT-DC group and higher than those of the IC group up to 35 days, drop abruptly at 49 days, to an even lower level than that of the IC group. This suggests that the impact of IC vaccine multiplication was even more

deleterious to immune protection when the vaccine strain was released to non IBD vaccinated chickens.

When elaborating a vaccination program for broiler flocks, we should not only consider the impacting diseases in our geographic area, but also choose the vaccines which do not negatively interfere on each other vaccine uptake.

ACKNOWLEDGEMENTS

Aurora Romero from x-OvO Limited performed the IB PCR.

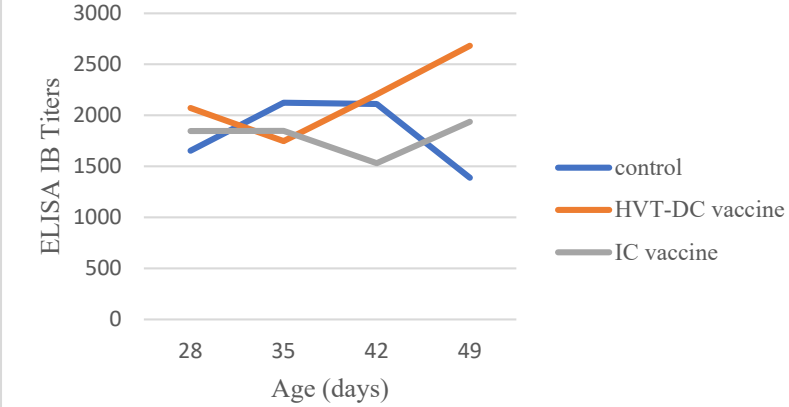
The RNA extraction, purification, VFS reaction, and analysis were performed by Rapid Genomics LLC.

REFERENCES

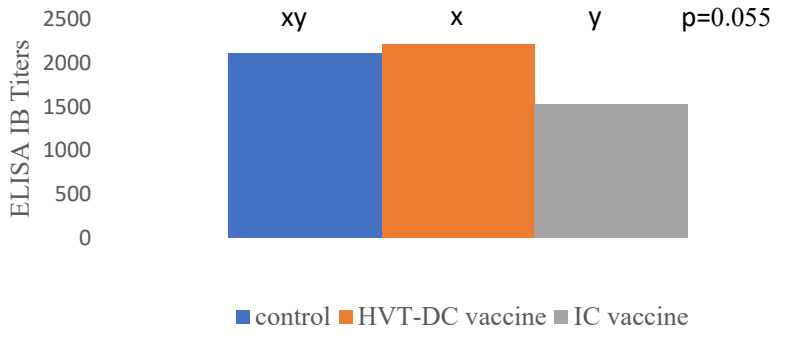
1. Lupini, C., G. Quaglia, G. Mescolini, E. Russo, R. Salaroli, M. Forni, S. Boldini, and E. Catelli. Alteration of immunological parameters in infectious bronchitis vaccinated–specific pathogen-free broilers after the use of
different infectious bursal disease vaccines. Poult. Sci. 99:4351–4359. 2020.

2. T.P. van den Berg T.P., N. Eterradossi, D. Toquin and G. Meulemans. Infectious bursal disease. Rev. sci. tech. Off. int. Epiz., 9 (2), 527-543. 2000.

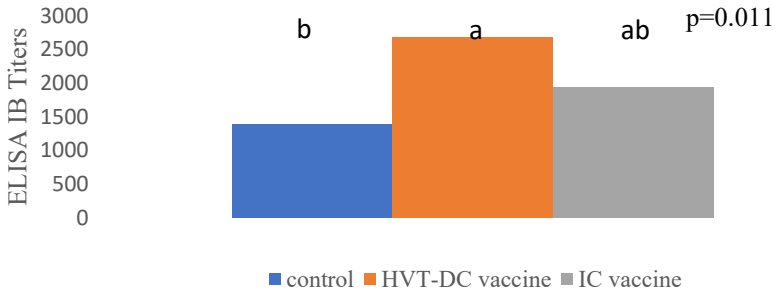
Graph 1. Evolution of IB titers per group



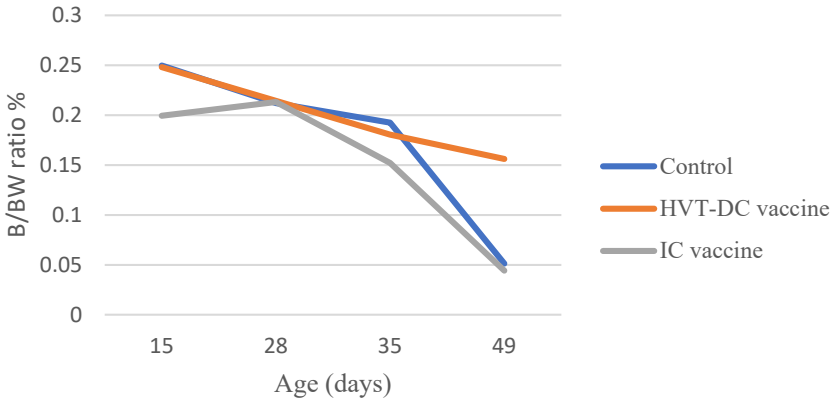
Graph 2. 42 days IB titers per group



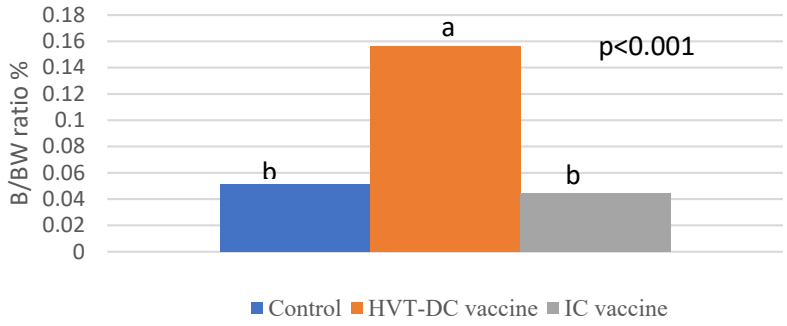
Graph 3. 49 days IB titers per group



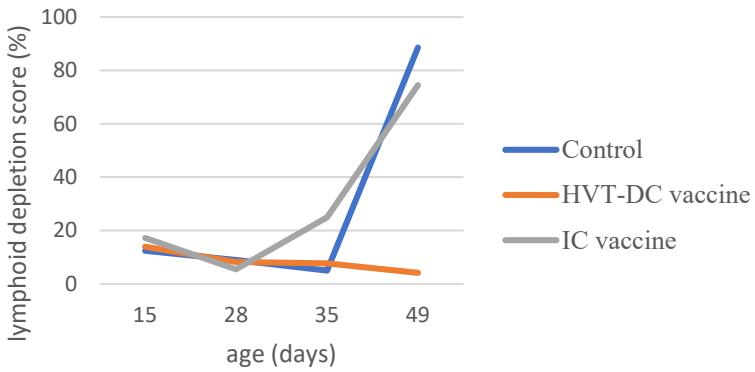
Graph 4. Evolution of B/BW ratio per age and per group



Graph 5. 49 days B/BW ratio per group



Graph 6. Evolution of lymphoid depletion score per group



Graph 7. 49 days lymphoid depletion score per group

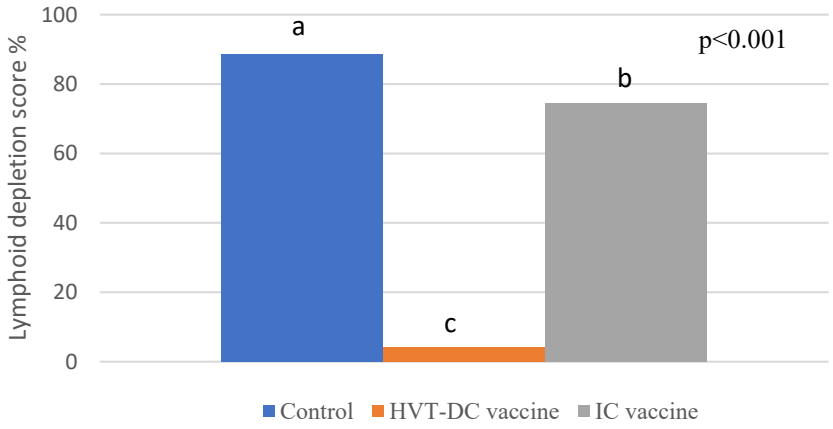


Photo 1. IC-vaccine 49 days lymphoïd depletion score: 77.5%.

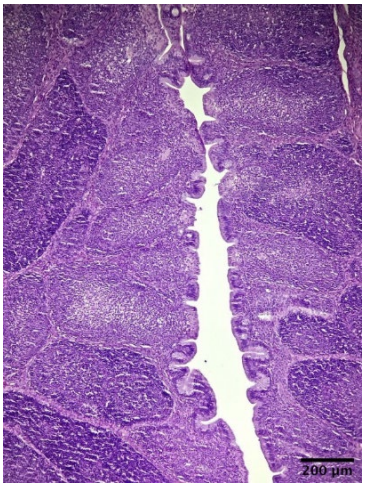
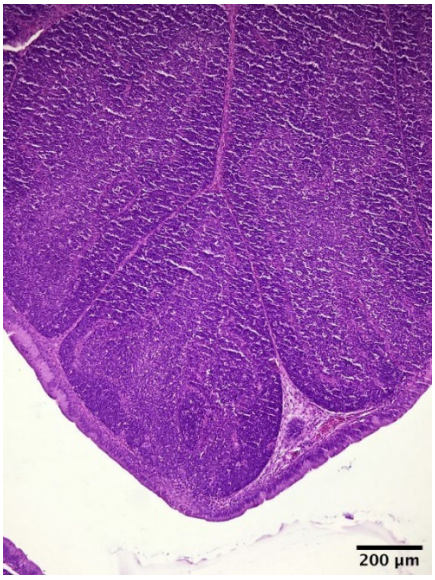


Photo 2. HVT-DC vaccine 49 days lymphoid depletion score: 3.8%.



LITERATURE REVIEW: MELOXICAM USE IN TURKEY POULTS

C. Caughlin[A], T. Girard[B], and H. Maloney[C]

[A]University of Calgary, Faculty of Veterinary Medicine, Calgary, AB T2N 4Z6
[B]Prairie Livestock Veterinarians, Red Deer, AB T4P 2T4
[C]Prairie Livestock Veterinarians, Red Deer, AB T4P 2T4

SUMMARY

In North America, turkey poult conditioning is performed on day-old poults at the hatchery. Producers may elect for various conditioning procedures based on the needs of their flock. The objective of conditioning treatments is to optimize bird welfare by reducing injury to anatomical structures prone to damage, remove anatomical features that are likely to damage other birds, and decrease stress from the associated injuries (1,2). In addition to improved welfare, poult conditioning is important for optimal carcass grading as scratches and lesions at slaughter will negatively impact grade, and ultimately reduce producer profit (3). Beak trimming is the most common treatment among hens and toms, while hens can additionally receive toe trimming. Toms may also undergo snood trimming (4). Investigations looking at the impacts of poult conditioning concur that there are positive economical and welfare implications to these treatments. However, there are inconsistencies reported assessing the level of sensation and discomfort to poults post-treatments. In North America, codes of practice are in place to ensure poult conditioning ensures welfare standards. Within these codes of practice, there are no regulations to provide analgesia at the time of poult conditioning. After poults receive these hatchery treatment(s), they are transferred to the farm and placed in a barn. During the first ten days after placement, turkey poults require vigilant care to ensure they thrive. This critical time is referred to as brooding. One factor that contributes to early mortality during the brooding period is failure to thrive: a delay in poults accessing or finding food and water, resulting in starvation and dehydration, leading to death. There are several factors that contribute to failure to thrive. One contribution may be related to pain or discomfort in poults after hatchery conditioning. Poults experiencing discomfort sensation may be reluctant to walk to feed and water. If post-conditioning analgesics are provided, there is potential to mitigate discomfort associated with these treatments, and reduce failure to thrive mortality. To date, there are no licensed non-steroidal anti-inflammatory (NSAID) pharmaceuticals for use in turkeys within North America. Future investigations may mirror the approval process of other production animal species to establish and approve NSAID safety, meat-withdrawal, and efficacy in turkeys.

Beak trimming. Beak trimming is performed in day-old poults to prevent aggressive pecking and cannibalism between flock-mates. Historically, a hot blade was used to instantly remove the tip of the beak while cauterizing the cut end. Current methods include microwave and infrared treatments (3). Infrared treatment exposes the tip of the beak to infrared light, preventing the underlying tissue from regrowth. Tissue will begin to necrose with sloughing of the beak to follow two weeks post-trimming. Assessment of the impacts of beak trimming measured body weight, feed intake, pecking force, and mortality in beak-trimmed versus controlled birds. The findings implied there was minimal difference in activity and preening behaviors in trimmed versus controlled birds. There was a marginal increase in time spent resting in beak-trimmed poults on day one, but beak trimmed poults spent more time walking compared to controlled birds by day ten. Beak trimmed birds had increased pecking force compared to their controlled counterparts. The author suggests the increased pecking force is correlated to some level of sensation during the sloughing process, however does not indicate this is related to pain. The most notable finding of this investigation is the reduced mortality due to aggression in beak-trimmed birds. Beak trimming is essential to maintain turkey welfare and minimize mortality due to aggression. This investigation suggests that this procedure is not painful and does not negatively impact turkey production (5).

Toe trimming. Toe trimming at the hatchery is commonly performed on turkey hens (4). At one day of age, the tips of the three forward facing digits (II, III, IV) are trimmed to inhibit future nail growth. The long-term objective of toe trimming is to prevent

carcass scratching between birds. Lesions associated with scratching decrease bird welfare (6). Toe trimming methods have evolved from hot blade trimming, to the current, and most common, method of microwave claw processing. While both methods are acceptable, microwave claw processing is the preferred recommendation (1). Hot blade trimming removes the distal end of the digit with a hot blade. In addition, hot blade trimming simultaneously cauterizes the end of the digit. This method reduces blood loss when compared to the earliest trimming method of surgical shears. Negative impacts of hot blade trimming are reported as shock, delayed growth, and pain. Microwave claw processing was introduced in the early 1990s and has become the most widely used toe trimming method. The tips of the digits are exposed to approximately 30 seconds of microwaves, preventing repair and regrowth of the nail. The claw will necrose and slough off in one to three weeks post-treatment (7). There are contrasting findings evaluating the post-trimming impacts on poults. Investigations reported minimal difference in mortality, feed intake, and body weight in trimmed versus non-trimmed poults (8,9). In contrast, another study found toe-trimmed poults spent more time resting and sitting, with less time at the feeders and drinkers, compared to their non-trimmed counterparts. Histological evaluation of trimmed toes found evidence of inflammation: infiltration of heterophils, edema, hemorrhage, and necrosis of the epidermis at day 0. The histological evaluation also determined that the site of tissue sloughing had an open epithelial layer, the physical barrier to the environment, until day eight post-trimming (6). There is evidence to suggest the removal of the distal part of the toes result in increased slipping leading to tibial rotation in toms, resulting in increased culling (10). Whether or not poults experience pain or discomfort post-toe trimming is inconclusive. The sensation of inflammation and level of bird comfort requires further investigation.

Failure to thrive. Early poult mortality failure to thrive describes a non-infectious mortality in poults 1-14 days of age (Figure 1) due to lack of nutrients and water consumption (11). On farm, these poults will appear unthrifty, with ruffled feathers, and a low head carriage (Figure 2). Post-mortem examination of failure to thrive poults is typically non-specific; however, evidence of litter-eating is a common finding (Figure 3). At the time of hatch, poults have an internal yolk sac that serves as their only source of energy and nutrients until feed is offered (12). Hatcheries may alleviate the gap in available nutrients by providing poult electrolyte pucks during transport, however variability in hatching time, conditioning treatments, and excess time in transport are stressors that can contribute to glucose dysregulation (13,11). Once placed on-farm, it is essential that poults efficiently access feed and water to replenish spent energy stores. Feed and water are immediately available to poults when placed on-farm, however sensation due to inflammation from hatchery conditioning, such as toe trimming, may prevent poults from accessing these critical resources, leading to starvation, and ultimately death. Reluctance to move due to pain or discomfort brought on by poult conditioning treatments requires further investigation to determine if pain control administered at placement could mitigate discomfort associated with these treatments, and ultimately decrease early mortality due to failure to thrive.

Analgesia. To date, there are no pain or anti-inflammatory pharmaceuticals licensed for use in turkeys within North American. Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) commonly used veterinary medicine, including use in other production animal industries, to treat pain and inflammation. There is limited literature available assessing pain control using NSAIDs in turkeys. One investigation assessed intravenous administration of three different NSAID to turkeys. Salicylic acid, flunixin, and meloxicam were found to have different half-lives with salicylic acid having the longest half-life, followed by meloxicam, with flunixin having the shortest. The author suggests that birds may not be able to conjugate salicylic acid with glycine, an essential step in the metabolic pathway (14). Intravenous administration of NSAIDs is not practical to the commercial turkey setting and requires a significant level of operator training. Alternative routes of administration must be explored to determine the most efficient and effective method. Licensed meloxicam pharmaceuticals are available for use in beef and dairy cattle, swine, and sheep (15). In other livestock species, analgesia is required for painful procedures, such as piglet castration (16). Other production animal industries have approved the use of meloxicam, instituting pain mitigation by providing analgesia and improve animal welfare.

CONCLUSION AND APPLICATIONS

The degree to which poult conditioning causes pain requires further investigation. There is published evidence that toe trimming causes inflammation, a notable welfare concern. The fact that there is no pain control available for use in Canadian commercial

poultry is alarming and a significant concern to the social licensing of the poultry industry. Establishing the use of non-steroidal anti-inflammatory drugs, specifically meloxicam, in turkey poults would have a practical application to the greater poultry industries. There may be applications beyond this investigation for anti-inflammatory use: Reovirus, osteomyelitis, and tibial dyschondroplasia are a few examples. Exploring meloxicam use in turkey poults will provide insight into areas for improved welfare and mortality management.

REFERENCES

1. National Farm Animal Care Council: Ottawa, ON, Canada. Code of practice for the care and handling of hatching eggs, breeders, chickens, and turkeys; 2016.
2. National Turkey Federation: Washington, DC, USA. Animal care best management practices for the production of turkeys; 2008.
3. Schwean-Lardner K, Anderson D, Petrik M, Torrey S, Widowski TM, Kitts B, Gardner J. *Code of practice for the care and handling of hatching eggs, breeders, chickens, and turkeys: Review of scientific research on priority issues.* National Farm Animal Care Council: Ottawa, ON, Canada; 2013
4. van Staaveren N, Leishman EM, Adams SM, Wood BJ, Harlander-Matauschek A, Baes CF. Housing and management of turkey flocks in Canada. *Animals.* 10:1159; 2020. http://doi:10.3390/ani10071159
5. Schwean-Lardner K, Fiss T, Struthers S. Impact of turkey beak treatment on welfare and production. [Unpublished]. College of Agriculture and Bioresources, University of Saskatchewan; n.d.
6. Fournier J, Schwean-Lardner K, Knezacek TD, Gomis S, Classen, HL. The effect of toe trimming of behavior, mobility, toe length and other indicators of welfare in tom turkeys. *Poult. Sci*. 94:1446-1453; 2015. http://dx.doi.org/10.3382/ps/pev112
7. Gorans MS. Methods and apparatus for declawing poultry. U.S. Patent 5,195,925; 1993
8. Carver DK, Fetrow J, Gerig T, Krueger KK, Barnes HJ. Hatchery and transportation factors associated with early poult mortality in commercial turkey flocks. *Poult. Sci.* 70:1818-1825; 2002. http://doi.org/10.1093/ps/81.12.1818
9. Schwean-Lardner K, Fournier J, Gomis S, Knzezacek T, Classen HL. n.d. Toe treatment in turkeys: Impacts on production and welfare [Unpublished]. College of Agriculture and Bioresources, University of Saskatchewan; n.d.
10. Fournier J, Schwean-Larnder K, Knezacek TD, Gomis S, Classen HL. The effect of toe trimming on production characteristics in heavy turkey toms. *Poult. Sci*. 93:2370-2374; 2014. http://doi.org/10.3382/ps.2014-04044
11. Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DD. *Diseases of Poultry*. 14th ed, Vol 1. American Association of Avian Pathologists; 2020.
12. Roehrig C, Torrey S. Mortality and early feeding behaviour of female turkey poults during the first week of life. *Front. Vet. Sci*. 6:129; 2019. http://doi.org/10.3389/fvets.2019.00129
13. Turner KA, Applegate TJ, Lilburn MS. Effects of feeding high carbohydrate or high fats diets. 1. Growth rate and metabolic status of the posthatch poult following immediate or delayed access to feed. *Poult. Sci*. 78:1573-1580; 1999. http://doi.org.10.1093/ps/78.11.1573
14. Baert K, De Backer P. Comparative pharmacokinetics of three non-steroidal anti-inflammatory drugs in five bird species. *Comp. Biochem. Physiol.* 134:1, 25-33; 2003. http://doi.org. 10.1016/s1532-0456(02)00184-9
15. Boehringer Ingelheim. *Compendium for Veterinary Products – Canada edition: Meloxicam 20 mg/mL solution for injection*. Animalytix; 2021.
16. National Farm Animal Care Council: Ottawa, ON, Canada. Code of practice for the care and handling of pigs; 2014.

Figure 1. An example of mortality trends due to failure to thrive.

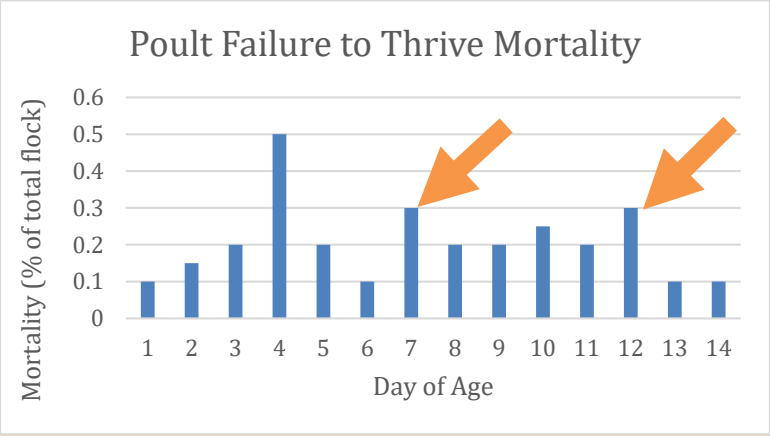
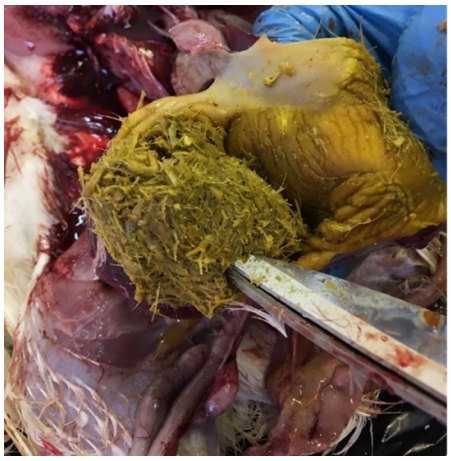


Figure 2. A 12-day old turkey poult with non-specific clinical signs of failure to thrive.
Photo credit: Dr. Teryn Girard

Figure 3. Evidence of litter eating on a post-mortem examination of a 12-day old turkey poult.
Photo credit: Dr. Teryn Girard



SEEDER BIRD RESEARCH- DEVELOPING THE BEST CHALLENGE MODEL

C. Clark[A], K. Gao[A], and P. Groves[A]

[A]The University Sydney, Sydney School of Veterinary Science, Sydney Australia

SUMMARY

Salmonella infection challenge models have been developed for research by oral, intraclocal, intratracheal, intraocular, navel and aerosol administration (1). This study was developed to assess the seeder challenge model in immature layers. In line with current vaccination procedures followed in Australia using a live *Aro-A* deletion *S. enterica* serovar Typhimurium vaccine. Commercial layers were followed out to 35 days of age to assess the success of using a seeder model to replicate the transfer of S. Typhimurium between young commercial layers and the effectiveness of the vaccine protocol used.

INTRODUCTION

To successfully develop a challenge model to simulate actual infection as seen in production practices, a seeder challenge model was proposed and previously shown to be an effective method to infect poultry (2,3). However, applied in this way to young layers is novel. This pilot study was developed with the objective to decipher the most successful challenge method. A second objective was to determine the base line effectiveness of the vaccine against the seeder challenge method. Once established, the methodology could be used to assess any confounding effect of a live *Salmonella* vaccine protection against *Salmonella*. Identifying the best challenge period; day 0 or week 3 in young birds will set the basis for a successful challenge and control with the current vaccine programs available in Australia.

MATERIALS AND METHODS

The intention to demonstrate if seeding birds at day old or three weeks will achieve colonization of the control birds and if the vaccination protocol demonstrates protection at this point (a colonization inhibition effect from the oral vaccine is expected following application). Pilot study design (looking at different challenge times under vaccination): live *Aro-A* deletion *S. enterica* serovar Typhimurium vaccine administered by coarse spray at day 0 and orally (gavage) at day 19 at single label dose rate.

There were two pens allocated for each group of eight birds (day old layer chicks). Pens for groups 1 and 3 were separated from groups 2 and 4 and biosecurity practices enacted between these to prevent cross contamination before the three-week challenge. Seeder birds were housed two per pen in suspended cages in each challenge pen for five days. The seeders were given an oral inoculation with 10^6 live *S.* Typhimurium. The seeder birds were infected at day 0 or at week 3. After five days the seeder birds were released into the pens. Cloacal swabs were collected at 10, 17, 24, 31 with ceca collected 35 days of age and cultured for the presence of *S.* Typhimurium.

RESULTS

Horizontal transmission from the seeder birds resulting in infection of the challenge birds is shown in Table 1. Seeder birds challenged at day 0 clearly showed better uptake for unvaccinated and vaccinated birds in this trial. The day 0 coarse spray vaccination program used in this study was unsuccessful against the seeder challenge at day 0 with a non-significantly different proportion of vaccinated and control birds returning a positive cloacal swab at each sampling point. Oral vaccination at 19 days did not provide any further protection against challenge from day 0.

The week 3 challenge achieved colonization in only three out of 16 control birds (19%) at 31 days and only two birds from 16 birds at day 35 from the ceca. This was not significantly different from the vaccinated group. The colonization of the seeder birds at 3 weeks was successful (7/8 birds with positive cloacal swabs at four days post infection) but this did not transmit effectively to the treatment birds.

DISCUSSION

Horizontal transmission results from seeder birds have been shown to facilitate gradual intestinal colonization (4). Isolation of *S.* Typhimurium from the

cloacal swabs of the control and vaccinated birds showed that there was consistent successful uptake by day 0 challenged birds. Young chicks (under three weeks of age) are regarded as much more susceptible to *Salmonella* infection than older birds and an infection establishing at this early age can allow continuous presence of the organism for long periods (1). Overall, the study demonstrated that the model of using seeder birds from day 0 could successfully horizontally transmit *S.* Typhimurium mimicking an early natural infection. Although infection of the seeders in cages at three weeks of age was successful, this did not transmit successfully to the birds on litter at three weeks of age. The day 0 administration of the live *Aro-A* deletion *S. enterica* serovar Typhimurium vaccine was overpowered by this challenge method.

REFERENCES

1. Gast, R.D. and Porter, R.E. Jr., Salmonella Infections. In: Diseases of Poultry. E. Swayne, ed. John Wiley & Sons, Inc. pp. 719-737. 2020.
2. Cox, N.A., A.A. Oladeinde, K.L. Cook, G.S. Zock, M.E. Berrang, C.W. Ritz, and A. Hinton. 2020. Evaluation of several inoculation procedures for colonization of day-old broiler chicks with Salmonella Heidelberg. Poultry Science 99:1615-1617.
3. Cox, N.A., J.S. Bailey, and M.E. Berrang. 1996. Alternative routes for Salmonella intestinal tract colonization of chicks. J Appl Poult Sci. 5;282-288.
4. Muir, W. I., W. L Bryden, and A. J. Husband. Comparison of Salmonella typhimurium Challenge Models in Chickens.

Table 1.

Pilot Study Treatments			Positive Results				
N= 16 per treatment	**Description**	**Challenge**	**10 Days Cloacal Swab**	**17 Days Cloacal Swab**	**24 Days Cloacal Swab**	**31 Days Cloacal Swab**	**35 Days Cecal Samples**
Control	ST Challenge	Day 0	16	16	14	12	10
Control	ST Challenge	3 Weeks	0	0	0	3	2
Vaccinated	ST Challenge	Day 0	15	16	15	15	11
Vaccinated	ST Challenge	3 Weeks	0	0	0	0	1

Table 2.

Seeder Birds Unvaccinated			Positive Results		
N= 4 per treatment	**Description**	**Challenge**	**4 Days Cloacal Swab**	**31 Days Cloacal Swab**	**35 Days Cecal Samples**
Control	ST Challenge	Day 0	4	3	4
Control	ST Challenge	3 Weeks	0	4	2
Vaccinated	ST Challenge	Day 0	4	3	4
Vaccinated	ST Challenge	3 Weeks	0	3	3

BROILER PROTECTION AND PERFORMANCE TRIALS OF THE NEWEST HVT-IBD RECOMBINANT VACCINE AGAINST AL2 AND GROUP-6 IBDV CHALLENGES

K. Cookson[A], M. Da Costa[A], J. Dickson[A], and J. Schaeffer[A]

[A]Zoetis—U.S. Poultry, Durham, NC

INTRODUCTION

AL2 viruses are the most prevalent variant IBDV type in the broiler industry. They have been recovered from about half of all flocks sampled over the past decade (1). Group-6 viruses, while a more antigenically diverse group of viruses than AL2, have also demonstrated an ability to override high Del-E type antibodies in progeny challenge studies (2). Previous studies comparing three HVT-IBD recombinants in SPF leghorns showed that there could be significant differences in early classic and variant (AL2) protection (3). This paper describes the results of three recombinant vaccine studies conducted in SPF broilers challenged with either AL2 or a Group-6 variant IBDV. It also introduces the first field trials comparing bird performance of the newest HVT-IBD (Vaccine A) to one already on the market.

MATERIALS AND METHODS

Isolator studies. Ross 708 broilers with no antibodies to IBDV were *in ovo* vaccinated with full doses of HVT-IBD vaccines (or not vaccinated—Controls) then allocated to five isolators each. Studies 1 and 2 compared AL2 protection of the newest HVT-IBD vaccine A with challenge at 19 days and 3.5 EID50 per bird or 18 days and 3.0 EID50 per bird, respectively. Study 3 compared Group-6 protection of all three HVT-IBD vaccines at 19 days and 3.0 EID50. All studies were terminated seven days post challenge. In all three studies, non-vaccinated, non-challenged controls are used to establish protection as any bird that is less than two standard deviations below the mean bursa to body weight ratio (B:BW) of the negative controls. All hypotheses were conducted at the $p \leq 0.05$ level of significance.

Field trials. Two companies in two different geographical locations conducted trials comparing their current HVT-IBD program (Vaccine B) to new HVT-IBD vaccine A using a week-on/off type design. Trial A was conducted in 31.8 million Ross 708 broilers and Trial B was conducted in 20.7 million Cobb 500s. Bursa surveys were done in flocks 18-30 days of age for histopathology and PCR analysis. Performance and condemnations were recorded and compared.

RESULTS

HVT-IBD Vaccine A broilers showed 70% and 78% protection against the AL2 challenge (see Table 1). The challenge "take" was similar whether given at 18 or 19 days of age and at a challenge dose of 3.0 or 3.5 EID50. In the Group-6 challenge study, HVT-IBD Vaccine A protection was 48% compared to HVT-IBD Vaccines B and C at 55% and 25%, respectively. Vaccine C bursas were significantly smaller than Vaccines A and B.

In broiler trial A, the window of IBDV field challenge was estimated at 18-28 days and consisted mostly of Group-6 type challenge. There were consistent numerical advantages to the Vaccine A flocks in all performance parameters including standard cost and 2/3 of a point in adjusted feed conversion rate (FCR) (see Table 2). In broiler trial B, the window of field challenge was estimated at 22-28 days and consisted mostly of AL2 type challenge. Less complete performance information was obtained but Vaccine A flocks showed a full point of improvement in adjusted FCR.

DISCUSSION

Previous SPF leghorn studies have demonstrated there can be differences in early IBDV protection of various recombinant HVT-IBD vaccines, depending on the virus type (3,4). Early onset of active immunity is crucial as maternal antibodies wane—especially where broilers are being raised in high (and variant) challenge environments. Transitioning to an SPF broiler model, studies 1 and 2 were able to demonstrate similar HVT-IBD Vaccine A protection levels against AL2 as in the SPF leghorn studies (3). Against a

Group-6 type challenge, Vaccines A and C gave ~50% protection while vaccine B yielded smaller bursas and about half the protection level.

In two field trials totaling over 50 million broilers, Vaccine A flocks demonstrated better performance values over Vaccine C flocks in two distinct geographic locations having different variant challenge types. In the 32 million bird trial A, Vaccine A flocks had 0.64 point lower adjusted FCR, while in the 21 million bird trial B, Vaccine A flocks demonstrated a full point lower adjusted FCR. These differences are of course well within the week-to-week "wobble" of any broiler complex. So the real question becomes how many millions of birds must be trialed for a 1 or 2 point FCR difference to be considered real? (Only descriptive statistical analysis was applied herein because enrolled farms were not randomized other than by their week of placement.) These trials were also conducted in summer months when IBDV and overall disease challenge tends to be at its lowest—probably making any differences in IBD impact even harder to measure (see low condemnations, Table 2).

In conclusion, HVT-IBD Vaccine A provides a third viable option in the single insert IBD recombinant space. In studies listed here and presented previously (3), HVT-IBD Vaccine A was the only vaccine to rank in the top tier on both Classic and Variant (AL2 and Group-6) IBDV protection before three weeks of age.

REFERENCES

1. Cookson, K., M. Da Costa, John Dickson and A. Barker. Update on IBD viruses infecting broilers today and how inactivated vaccines protect against different viruses in the AL2 family. Proc. Western Poultry Diseases Conference, Sacramento, CA. 2020.

2. Cookson, K., M. Da Costa, J. Dickson, B. Ladman and J. Schaeffer. Field trial comparing different vaccination programs on broiler progeny protection against a paenl of variant IBD viruses. Proc. Western Poultry Diseases Conference, Virtual Meeting. 2021.

3. Brown, A., L. Taylor, M. Bosserd, J. Dickson, J. Embrey and A. Hartman. Early onset of immunity and duration of immunity of a recombinant HVT-IBD vaccine against virulent, variant and very virulent IBD. Proc. AAAP Meeting, Virtual Meeting. 2020.

4. Gelb, J., D. Jackwood, E. Brannick and B. Ladman. Efficacy of recombinant HVT-IBD vaccines administered to broiler chicks from a single breeder flock at 30 and 60 weeks of age. Avian Dis. 60:603-612. 2016.

Table 1. Summary of three isolator challenge studies in HVT-IBD vaccinated broilers.

Treatment	Study 1		Study 2		Study 3	
	AL2 at 19 days 3.5 EID50		AL2 at 18 days 3.0 EID50		Group-6 at 19 days 3.0 EID50	
	B:BW	Protection	B:BW	Protection	B:BW	Protection
Negative control	1.887a	-	2.003a	-	1.812a	-
Challenge control	0.592c	5%b	0.645c	5%b	0.615d	0%b
Vaccine A HVT-IBD	1.462b	70%a	1.382b	78%a	1.125b	48%a
Vaccine B HVT-IBD	-	-	-	-	0.846c	25%a
Vaccine C HVT-IBD	-	-	-	-	1.340b	55%a

Table 2. Summary of large broiler field Trial A in a multi-complex site with predominantly Group-6 type challenge.

Treatment*	# Placed	ADG	Body Wt.	FCR	Adj. FCR	% Livability	% Cond.	Std. Cost
Vaccine A HVT-IBD	13.0M	0.1495	8.0259	1.7585	1.7484	96.0666	0.0368	20.1354
Vaccine C HVT-IBD	18.8M	0.1490	8.0229	1.7635	1.7548	95.893	0.0376	20.2677
Vaccine A advantage	-	0.0005	0.0030	0.0005	0.0064	0.1729	0.0008	0.1323

*Besides the recombinant HVT-IBD vaccine, all flocks also received a half dose of mild IBD vaccine *in ovo*.

A REVIEW AND COMPARISON OF HOUSING SYSTEMS FOR COMMERCIAL LAYERS

G. Cutler

Cutler Associates International, P.O. Box 1042, Moorpark, CA 93020

SUMMARY

An overview of commercial egg layer housing systems will be reviewed and compared. In the 1960s, there were over 12000 egg producers in the United States, most were small, geographically dispersed operations that sold eggs locally. In the 1980s the industry underwent significant consolidation. Some of the top egg-producing States in the 1980s included California, Pennsylvania, Texas, Indiana and Florida. Currently, there are less than 200 large-scale commercial egg producers in the United States. In the last decade, the top egg-producing States include Iowa, Ohio, Pennsylvania, Indiana, Texas, and California. Factors such as Proposition 2, and consolidation of layer companies have led to a reduction in egg production in California.

Current management systems in the layer industry utilize vertical integration, which has facilitated increased economic benefits and has allowed companies to become more efficient. Most companies raise their own pullets from day-of –age until approximately 16-18 weeks of age in a grow facility. Pullets are then moved to the lay house and kept until the end of the flock cycle. Molting is done less frequently in modern egg production systems and egg-producing flocks are mostly kept for one cycle, until 85-90 weeks of age.

Egg production facilities in 1930s and 1940s consisted largely of colony wire cages while modern facilities are often comprised of multiple rows of multistory complexes, often with 100,000-200,000 chickens per building. Housing in California must meet Proposition 2 requirements. Proposition 2 requires that on and after January 1, 2020, a farm owner or operator in California shall "not confine an egg-laying hen in an enclosure with less than 1 square foot (144 square inches) of usable floor space per egg-laying hen."[1] Commercial egg production systems in California are now all cage-free operations, most of which are multi-tiered aviaries. A cage-free housing system is defined as a "controlled environment for egg-laying hens within which the egg-laying hens are free to roam unrestricted, are provided enrichments that allow them to exhibit natural behaviors, including, at a minimum, scratch areas, perches, nest boxes, and dust bathing areas, and within which farm employees can provide care while standing within the hens' usable floor space."

REFERENCE

1. California Legislative Information. AB-3021 Farm animals: egg-laying hens: confinement.(2017-2018) https://leginfo.legislature.ca.gov/faces/billTextClient.xhtml?bill_id=201720180AB3021.

INFECTIOUS CORYZA: A CONCERTED EFFORT TOWARDS A NOVEL CLASSIFICATION METHOD FOR *AVIBACTERIUM PARAGALLINARUM*

A. da Silva[A], R. Buter[B], Remco Dijkman[B], A. Feberwee[B], J. Mills[C], R. Beckstead[C], Y. Huberman[D], R. Malena[D], F. Paolicchi[D], M. Jonas[E], E. Soriano-Vargas[F], and R. Gallardo[A]

[A]Poultry Medicine laboratory, School of Veterinary Medicine, University of California, Davis
[B]Royal GD Animal Health, Deventer, the Netherlands
[C]Scientific Support and Investigation Unit, Ceva Animal Health, USA
[D]National Institute of Agro-Technology (INTA), Balcarce, Argentina
[E]PT Medion Farma Jaya, Indonesia
[F]Center of Investigation and Advanced Studies in Animal Health, School of Veterinary Medicine and Zootechnics, National Autonomous University of Mexico

SUMMARY

Infectious coryza is an upper respiratory disease of chickens caused by the bacterium *Avibacterium paragallinarum*. Currently, there are two classification methods for *A. paragallinarum* using hemagglutination inhibition tests. The Page scheme divides strains into serogroups A, B and C, while the Kume scheme subdivides serogroups A, B and C into 9 serovars: A-1 to A-4, B-1, and C-1 to C-4. Both assays are complex, expensive, and performed in very few laboratories. To overcome this challenge, we developed a molecular classification method that corresponds to the Kume scheme targeting the HMTp210 gene. This gene codes for a membrane protein with hemagglutinating capability. The phylogenetic analysis is composed of two parts: (1) one portion of the gene that classifies all strains into serogroups A, B and C, and (2) the entire gene that attempts to classify strains into serovars A-1 to A-4, B-1, and C-1 to C-4.

INTRODUCTION

Infectious coryza is a bacterial upper respiratory disease caused by *Avibacterium paragallinarum*, a Gram-negative bacterium of the *Pasteurellaceae* family. The disease is typically characterized by conjunctivitis, ocular discharge, swelling of the infraorbital sinuses, facial edema, and sneezing. More severe clinical signs might be observed if infection is complicated by other respiratory pathogens (1).

Serotyping continues to be the gold standard for *A. paragallinarum* classification. Two methods using hemagglutination inhibition tests have been described. The Page scheme classifies strains into serogroups A, B and C. Although the Page scheme is easier to execute compared to Kume, many isolates are non-typable. The Kume scheme uses potassium thiocyanate-treated red blood cells and is therefore more laborious. The Kume scheme is more sensitive for serotyping than Page, and strains are classified into serovars A-1 to A-4, B1, and C-1 to C-4 (2). Nevertheless, both serotyping methods are laborious, time consuming, and subjective. Because very few laboratories offer *A. paragallinarum* serotyping throughout the world, this method is also expensive since it involves shipping of live biological material internationally.

To overcome the *A. paragallinarum* classification challenges, genotyping methods based on genes that code for bacterial virulence factors have been tested (3,4). The most promising target so far is HMTp210, a gene of nearly 6.1 kbp that encodes for a 210 kDa protein. Because of its hemagglutinating activity, HMTp210 is likely the most important immunogenic antigen of *A. paragallinarum* (5). This study aims to compare the phylogenetic relationship between *A. paragallinarum* strains that belong to different serotypes with the goal of determining a more practical classification method than the serotyping assays currently used.

MATERIALS AND METHODS

A total of 125 HMTp210 sequences were obtained for phylogenetic analyses from GenBank or through a consortium between UC Davis and animal health companies in various countries throughout the world. Serotyping metadata were available for 23

sequences deposited on GenBank. The consortium contributed with 30 serotyped HMTp210 sequences, totalizing 53 sequences of known Kume or Page serotypes. The remaining 72 sequences were not serotyped but included in the phylogenetic analysis. Of the 53 serotyped sequences, 20 belonged to Page serotype A and 18 were serotyped using the Kume method (A-1 = 11, A-2 = 5, A-3 = 1, and A-4 = 1). Ten sequences were classified as either B (Page) or B-1 (Kume). Twenty-one were classified as Page serotype C; of these, 14 were classified using the Kume method (C-1 = 3, C-2 = 9, C-3 = 1, and C-4 = 1). One sequence from Taiwan was reported to cross-react to B and C antisera in the Page serotyping (4).

The HMTp210 sequences were aligned using the MAFFT plugin in Geneious Prime. Phylogenetic trees using partial or whole HMTp210 gene sequences were build using the maximum likelihood method based on the GTRGAMMAI model with 1,000 bootstraps using the RaxML plugin in Geneious Prime.

RESULTS AND DISCUSSION

A fragment of 1,249 bp targeting the 5' portion of the HMTp210 gene has successfully grouped Page serogroups A, B and C in phylogenetic analyses (4) (Figure 1A). However, we noticed that there were other regions of variability in the HMTp210 gene that could interfere with the clustering of the sequences, especially within serotype A strains. Using the entire HMTp210 gene (~6.1 kbp), a phylogenetic tree was constructed to assess the evolutionary relationship between different serotypes of *A. paragallinarum* (Figure 1B).

The distribution of sequences represented in red (n = 32) are identical using the partial and the whole HMTp210 methods (Figures 1A and 1B). This cluster includes sequences from Taiwan, the Netherlands, Colombia, and France that have been classified as C (n = 7), C-1 (n = 2), C-2 (n = 4), and C-4 (n = 1). The recombinant B/C strain from Taiwan is also in the red cluster, showing close relationship to the other sequences using the partial gene (Figure 1A) and is distantly related to others using the whole gene (Figure 1B). The classical strains H-18 (C-1), Modesto (C-2), and HP60 (C-4) are closely related and within this cluster, with nucleotide identities higher than 95% between each other in the whole HMTp210 gene. Moreover, the HMTp210 genes from Modesto and H-18 strains are 98.7% identical. These C strains are commonly present in commercial inactivated vaccines. These results suggest that there might be other genes or proteins with hemagglutinating capabilities that play a role in the Kume classification other than HMTp210. Nevertheless, it has been shown that serogroup C strains of different serovars induce cross-protection (3).

A second cluster of C sequences are represented in blue (Figure 1). The whole HMTp210 phylogenetic tree comprises a total of 32 sequences, all from the United States, of which four have been serotyped as C-2 (Figure 1B). Three sequences that cluster with B strains in the whole gene phylogenetic tree show relatedness to the American C strains (blue cluster) using the partial HMTp210 gene (Figure 1A). This result raises the suspicion of possible recombination events within HMTp210 between B and C strains as previously reported (4). The single C-3 sequence used in this study (represented in yellow) seems to have common evolutionary roots as the American C strains (represented in blue) but is distinct enough to be represented on its own branch (Figures 1A and 1B).

The A strains seem to be better distributed phylogenetically when using the whole HMTp210 gene rather than the partial gene. Three clusters are identified using the whole gene (Figure 1B, green, purple, and pink), whereas there is no clear pattern of distribution using the partial gene (Figure 1A). The green cluster in Figure 1B comprises 35 sequences, of which two were classified as A, 11 as A-1 and two as A-2. Most of the sequences from the green cluster are from the Netherlands, with a few sequences from China, Japan, UK, Spain, Colombia, and the US. One sequence from Florida seems distantly related due to problems with sequencing coverage, providing a skewed relationship with the other sequences. From the six strains represented in the purple cluster, three belong to serovar A-2 and one from serogroup B. Serovars A-3 and A-4 have only one representative each and cluster together in a separate branch (pink, Figures 1A and 1B).

In conclusion, genotyping using the integral or partial HMTp210 gene provides robust classification of *A. paragallinarum* strains into Page serogroups A, B, and C. For A strains, the whole HMTp210 gene phylogenetic analysis is more reliable and provides more insights into the evolutionary patterns of this specific gene. More serotyped isolates must be sequenced to provide strength to this classification method. The mismatches between serotypes and genotypes bring to light the reliability of the Kume and Page serotyping methods, which at times can be subjective due to cross-reaction between strains and antisera and due to the difficulties in performing the procedure. On the other hand, the role of other hemagglutinating proteins as virulence factors of *A.*

paragallinarum must be studied to better understand the relationship between the bacterial genome and serotyping. This study contributes to the rapid and accessible typing of *A. paragallinarum*, which is fundamental for the appropriate choice of vaccines for the control of infectious coryza in commercial poultry.

REFERENCES

1. Blackall P, Soriano-Vargas, E. 2020. Infectious coryza and related bacterial infections. In: Swayne DE, editor. Diseases of poultry. 14th ed. United Kingdom: Wiley-Blackwell. p. 890-906.

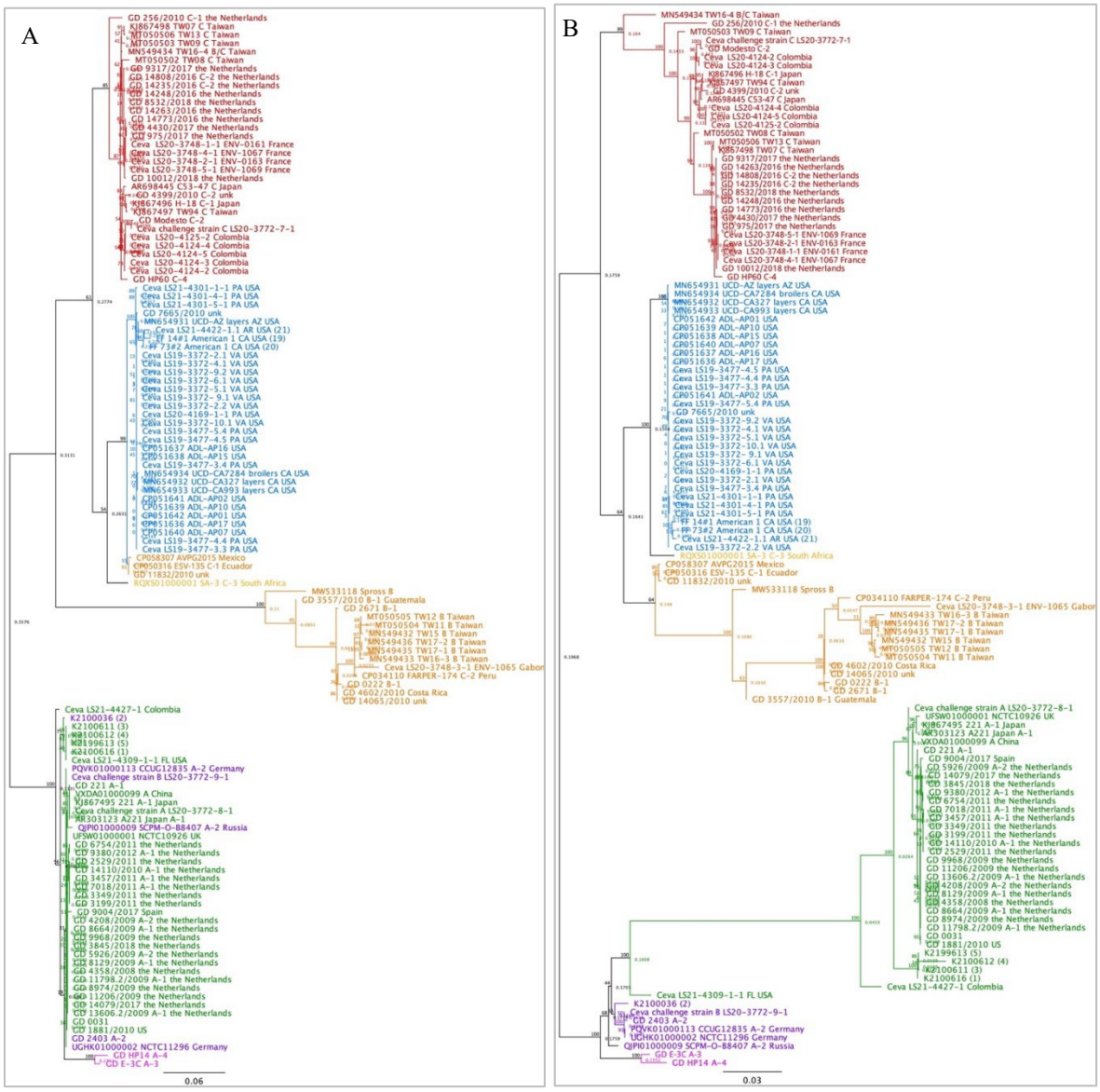
2. Blackall PJ. Infectious coryza: Overview of the disease and new diagnostic options. *Clin Microbiol Rev*. 12(4):627-632; 1999.

3. Gallardo RA, da Silva AP, Egaña-Labrin S, Stoute S, Kern C, Zhou H, Cutler G, Corsiglia C. Infectious coryza: Persistence, genotyping and vaccine testing. *Avian Dis*. 65:157-165; 2020.

4. Tan DH, Ou SC, Shien JH, Huang SW, Hsieh MK, Chang PC. Serotypes and hemagglutinin gene sequences of *Avibacterium paragallinarum* isolated in Taiwan. *Avian Dis*. 64(2):197-202; 2020.

5. Wang Y-P, Hsieh M-K, Tan DH, Shien JH, Ou SC, Chen CF, Chang PC. The haemagglutinin of *Avibacterium* paragallinarum is a trimeric autotransporter adhesin that confers haemagglutination, cell adherence and biofilm formation activities. *Vet Microbiol*. 174(3):474-482; 2014.

Figure 1. Phylogenetic trees using partial (A) or whole (B) HMTp210 genes of 125 Avibacterium paragallinarum strains. Fifty-three sequences have known serogroups or serovars and are indicated on the sequence name. Each color represents a cluster based on the phylogenetic tree using the whole HMTp210 gene (B).



FIELD EXPERIENCE - INFECTIOUS BRONCHITIS VACCINATION TAKES BEFORE AND AFTER VACCINE APPLICATION AND OPTIMIZATION OF SAMPLING METHODOLOGY

M. Da Costa, K. Cookson, T. Cummings, and J. Schaeffer

Zoetis, U.S. Poultry

INTRODUCTION

A broiler integrator in the Southeastern United States was on a Massachusetts (Mass) plus Georgia 08 (GA08) infectious bronchitis (IB) vaccination program, and during a routine IB vaccine takes evaluation, the veterinarian identified relatively low vaccine takes via real-time PCR of trachea swabs. The vaccine preparation/application and trachea sampling process were assessed to troubleshoot the low vaccine takes identified. The following survey shows how identifying and implementing corrective actions can significantly impact IB vaccine takes.

MATERIALS AND METHODS

Problem assessment. At the time of the first visit and vaccine takes evaluation, it was identified that the vaccine preparation conditions were outside of the recommended parameters. The water bath for vaccine thawing (GA08 is a frozen vaccine) and diluent temperatures were being set to temperatures used for *in ovo* Marek's/Vector vaccine preparation (80°F and 70°F, respectively). During this first evaluation, 15 tracheal swabs (general transport tubes with Amies agar) were collected per farm from 7-day old birds from 7 different farms.

Corrections implemented and follow-up assessment. After identifying the problems at the time of the first visit, corrections were implemented on vaccine preparation and sampling for takes procedures. Water temperatures for vaccine thawing and diluent were changed to 70°F and 45-50°F, respectively, according to Zoetis IB vaccine preparation recommendations best practices. Vials were pulled from the water bath before the ice plug had completely thawed (vaccines shouldn't come up to 70°F). Pulling the vials just before the ice plug completely thawed helped ensure that no vials were left in the water bath temperature for too long. On the sampling methodology for takes, whole individual tracheas were collected into Whirl-Pak® bags, frozen, and shipped with ice packs for next-day delivery. Fifteen birds (five to six days old) were sampled from each of 8 different farms.

Both tracheal swabs and tissue samples were processed for IB virus quantification by RT-PCR serotype-specific (Mass and GA08) according to the methodology described by Roh *et al*. 2013 (1). Average CT values and percent positives (CT<40) are presented.

RESULTS AND DISCUSSION

The first and second vaccine takes evaluation results are presented in Tables 1 and 2, respectively. Table 3 has a side-by-side comparison of the two evaluations performed, with the respective improvement rates for viral load and percent IB vaccine positives by serotype.

As shown on the results from the first visit, the higher thawing bath and diluent temperatures, along with a lack of emphasis to pull frozen vials before they completely thawed, were resulting in low GA08 takes, with only 40% of the birds showing a weak CT positive signal. This had presumably resulted in vaccine titer losses before it reached the chick. After adjusting water temperatures and thawing technique, all birds sampled were positive for the GA08 vaccine, improving the CT signal corresponding to nearly a 4 Log increase in vaccine virus take. The Mass serotype also saw a modest improvement in vaccine take (1 Log). This improvement was likely related to mixing the vaccine with a cooler diluent and switching from swab to trachea tissue samples. The disparity in vaccine takes between the freeze dried and frozen vaccines suggests that closer attention to proper thawing technique is necessary.

Another factor that cannot be disregarded is the differences in sample methodology between the two evaluations. On the first evaluation, the tubes with media were frozen and then thawed for testing. This could have caused a dilution of the viral load resulting in artificially higher CT values. Consequently,

sampling tracheal tissue might be more appropriate for an accurate vaccine takes evaluation.

In conclusion, IB vaccine takes testing is a good way of monitoring vaccine administration that can ultimately impact vaccine performance. Regular evaluations can uncover potential hatchery failures and compatibility of different vaccine serotypes.

REFERENCES

1. Roh, H.J., Hilt, D.A., Williams, S.M. and Jackwood, M.W., 2013. Evaluation of infectious bronchitis virus Arkansas-type vaccine failure in commercial broilers. *Avian Diseases, 57(2)*, pp.248-259.
TI-07248

Table 1. Summary of results at the time of the first IB vaccine take evaluation.

	Farm 1		Farm 2		Farm 3		Farm 4		Farm 5		Farm 6		Farm 7	
	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08
Mean CT value	31	36.5	30	36.3	29.7	36.2	28.7	35.6	31	39.5	31.2	39.4	33.4	39.4
% Positives	100	53	100	47	93	53	100	60	100	27	100	27	93	13
Age (days)	7		7		7		7		7		7		7	

Table 2. Summary of results of the second IB vaccine take evaluation after implementing vaccine preparation and trachea sampling correction measures.

	Farm 1		Farm 2		Farm 3		Farm 4		Farm 5		Farm 6		Farm 7		Farm 8	
	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08	Mass	GA08
Mean CT value	27.8	23.4	27.2	23.4	29.4	24.2	28.3	27.3	26	25.7	25.3	27.1	25.8	25.6	28.5	19.8
% Positives	100	100	93	100	100	100	100	100	100	100	100	100	100	100	100	100
Age (days)	6		6		5		5		5		5		5		6	

Table 3. Comparison of the two IB vaccine take evaluations performed, with the respective improvements in viral load and percent IB vaccine positives by serotype.

Serotype →	Mass			GA08		
	Before	After	Improvement	Before	After	Improvement
Mean CT value	30.7	27.3	10-fold (1 log)	37.6	24.6	>8,000-fold (4 log)
% Positives	98	99	↑1%	40	100	↑150%

CAUSES OF MORBIDITY AND MORTALITY OF PEAFOWL & PEAFOWL PHEASANTS AT ZOOLOGICAL INSTITUTIONS: A RETROSPECTIVE STUDY

M. Drozd[A], M. Dominguez[A], T. Morishita[A], D. McClure[A], and M. Garner[B]

[A] College of Veterinary Medicine, Western University of Health Sciences, 309 E. Second Street, Pomona, CA 91766
[B] Northwest ZooPath, 654 West Main Street, Monroe, WA 98272

SUMMARY

Many zoos have free-roaming peafowl and peafowl have been shown to transmit many diseases. It is important to know the causes of morbidity in peafowl since they have the ability to transmit diseases to zoological collection species.

A retrospective study was conducted to determine the cause(s) of morbidity and mortality in free-roaming peafowl in zoological institutions throughout the United States of America. Out of 334 birds included in this study, 32.6% died of traumatic injury and 31.1% died of infectious diseases. Of the 104 peafowl that died of infectious diseases, known causes were mainly bacterial and fungal in origin. Identifying possible cross-species infectious etiological agents could aid in evaluating overall zoo population health.

(A full-length article is currently in preparation for a peer-reviewed journal publication. Please contact primary author for more information.)

AN EMERGING *ENTEROCOCCUS CECORUM* OUTBREAK IN A BROILER INTEGRATOR IN THE SOUTHERN US: ANALYSIS OF ANTIMICROBIAL RESISTANCE TRENDS

G. Dunnam[A], J. Thornton[A], and M. Pulido-Landinez[A]

[A]Mississippi State University, College of Veterinary Medicine, Poultry Research and Diagnostic Laboratory

SUMMARY

Enterococcus cecorum is associated with vertebral osteomyelitis of the free thoracic vertebrae in chickens; however, there are reports of *E. cecorum* producing septicemic lesions and having a tropism for cartilages resulting in the presentation of femoral head necrosis and synovitis. This paper discusses the presentation of *E. cecorum* as it relates to an outbreak in a vertical integrator where the main lesions were septicemia. 85 laboratory accessions were analyzed as being positive for *E. cecorum*; of these, eighty-one were from broilers and four were from broiler breeders. The average age of broilers was approximately 20 days with a range of 15-31 days. The broiler breeders averaged approximately 25 weeks of age with an age range of 5-51 weeks. Three of the 85 isolates were isolated from the free thoracic vertebrae with the remaining being recovered from various other locations including: liver, hock/joint, femoral head/bone marrow, and pericardium. Antimicrobial sensitivities were only analyzed for broilers using WHONET Microbiology Laboratory Database Software. No isolates were pan-susceptible and 87.7% of isolates were noted to be resistant to $\geq$3 classes of antibiotics.

INTRODUCTION

Initially isolated from the gastrointestinal tract of chickens in 1983, *Enterococcus cecorum* is a facultatively anaerobic, gram-positive cocci (1). It was first described as normal flora in chickens *(Gallus gallus domesticus)*(2). However, over the past two decades, pathogenic strains of *E. cecorum* have emerged within the commercial poultry industry (2). Outbreaks of pathogenic *E. cecorum* causing vertebral and femoral osteomyelitis were first reported in 2002 in Scotland and the Netherlands with a vast array of reports from other countries following (2,3). It now appears that *E. cecorum* is regionally endemic (2). The most consistent lesion associated with pathogenic *E. cecorum* infection involves the free thoracic vertebrae (FTV) in the spinal column. The FTV sits cranial to the synsacrum and caudally to the notarium. This lesion is characterized by an inflammatory mass in the area of the FTV (2). This swelling extends dorsally to compress the spinal cord resulting in a clinical symmetrical paralysis in the individual bird. This lesion is referred to as "vertebral osteomyelitis, vertebral enterococcal osteomyelitis and arthritis, enterococcal spondylitis, and colloquially, 'kinky-back'" (2). However, kinky-back is also the term used to refer to malformation of the spinal column (spondylolisthesis) which is a developmental condition that results in the same outcome of symmetrical paralysis as enterococcal induced infection (3).

In addition to lesions affecting the FTV, *E. cecorum* has been associated with femoral head necrosis, osteomyelitis, pericarditis, perihepatitis, and splenomegaly (1,2). These lesions are due to a septicemic phase of the disease which can have variable mortality and morbidity and closely resembles other septicemias such as colibacillosis (2). While the organism has been isolated from yolk sacs from chicks prior to 14 days of age, there have been reports of increases in mortality and morbidity due to the septic phase within the first 2-3 weeks of age with other reports of infections in broilers at 3-4 weeks of age (2, 4). The bacteremia within the flock has been reported to increase with age suggesting that birds do not clear the infection efficiently (2). In addition, *E. cecorum* has been reported to have a predilection for cartilage and bone (4). Interestingly, most birds that become systemically infected with pathogenic *E. cecorum* do not show clinical signs of sepsis (3, 6).

There has not been a determination of the source of pathogenic *E. cecorum* involved with outbreaks in vertical integrators. Vertical transmission has not been definitively demonstrated as one study failed to recover pathogenic *E. cecorum* from the hatchery and from dead embryos; however, the chicks placed at two separate farms developed outbreaks of pathogenic *E.*

cecorum with identical genetic and phenotypic profiles (2,3,4). There has been hypothesis that pathogenic *E. cecorum* originates from the gastrointestinal tract and gains entry to circulation due to intestinal injury; however, disease has been recreated without gut lesions (3). While the full mechanism of infection may not be worked out, horizontal transmission is rapid, and lesions associated with *E. cecorum* have been observed after experimental oral and intravenous inoculation (2).

In prior studies, pathogenic *E. cecorum* isolates have shown resistance to a broad range of antibiotics (5). However, in one study most isolates were susceptible to ampicillin, high level gentamicin, chloramphenicol, and vancomycin (5). *E. faecalis* and *E. facium* possess low affinity penicillin binding proteins (PBPs) therefore making beta-lactam antibiotics less effective when used alone (2). In addition, the two mentioned *Enterococcus* species are also intrinsically resistant to achievable levels of aminoglycosides (2). Enterococci also can absorb folic acid from their environment making sulfonamides less efficacious (2). The purpose of this study was to describe the ongoing outbreak of *E. cecorum* in a vertical integrator and evaluate antimicrobial sensitivity patterns to determine trends between isolates.

MATERIALS AND METHODS

An accession list was built from the Poultry Research and Diagnostic Laboratory's accession system. These were to include *Enterococcus cecorum* isolates that were originally isolated in the PRDL microbiology laboratory from case submissions extending from January 1, 2021 to October 27, 2021 from the integrator experiencing the outbreak. *E. cecorum* identification was performed after 24 hours of incubation, by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Vitek® MS instrument (bioMerieux, Inc). Samples were prepared according to manufacturer recommendations. Antibiotic minimal inhibitory concentrations (MICs) for these isolates were obtained using the Avian1F Vet AST plate, Thermo Fisher® Sensititre® Complete Automated AST System following manufacturer recommendations. The Avian1F plate determines the MIC for each of the following antibiotics: amoxicillin (AMX), ceftiofur (TIO), clindamycin (CLI), enrofloxacin (ENR), erythromycin (ERI), gentamicin (GEN), neomycin (NEO), oxytetracycline (OXY), penicillin (PEN), sulfadimethoxine (SUD), spectinomycin (SPT), streptomycin (STR), tetracycline (TCY), trimethropim/sulfamethoxazole (SXT), and Tylosin (TYL). *Escherichia coli* (ATCC 8739) was used as a quality-control strain. The MICs were then entered into WHONET Microbiology Laboratory Database Software (WHO). MICs breakpoints used were obtained from Thermo Fisher, SWIN module (7), Clinical and Laboratory Standards Institute (8), and USDA (9). WHONET was used to establish the sensitivity trends per each isolate, including the percentage of resistant, intermediate, and susceptible isolates (%RIS multifile analyses), and the determination of the antimicrobial resistance profiles (ARPs) of each isolate.

RESULTS

General results. Eighty-five laboratory accessions were found matching the query. Of these, eighty-one were from broilers and four were from broiler breeders. The average age of broilers was approximately 20 days with a range of 15-31 days. The breeders averaged approximately 25 weeks of age with an age range of 5-51 weeks. Table 1.1 shows the site of isolation for the 85 isolates included in this study (most isolates were recovered from more than one site). Bacterial coinfection was identified in 35.7% of cases. 63.1% of cases were positive to avian reovirus (viral arthritis). Other comorbidities not listed included IBD, IBV, Rickets, and histomoniasis. Antimicrobial sensitivities were run on the isolates from broilers, n=81.

Antimicrobial sensitivity. Eighty-one isolates were tested for all antibiotics except for gentamicin, neomycin, and streptomycin in which seventy-eight samples were tested. No isolates (0%) were pan-susceptible; the two highest levels of resistance were to streptomycin and spectinomycin with values of 82.1% and 82.7%, respectively. The highest susceptibility was to enrofloxacin (79%), ceftiofur (84%), gentamicin, (85.9%), and amoxicillin (88.9%). A complete list of all antibiotics with sensitivities can be found in table 2.1.

Seventy-one (71) or 87.7% of isolates were found to be multi-drug resistant (resistant to $\geq$3 classes of antibiotics).

Resistance profiles can be found in table 2.2.

Age relationship. The isolates from broilers were divided by age into three categories: 15-19 days, 20-24 days, and 25+ days. The results were 32 isolates between 15-19 days of age, 37 between 20-24, and 10 greater than or equal to 25 days of age. The isolates from the youngest group were resistant to the most

antibiotics on average (6.2 antibiotics), followed by the 20-24-day old group (resistant to an average of 5.5 antibiotics). Isolates from the oldest group were resistant to an average of 4.6 antibiotics.

DISCUSSION

This is an example of an outbreak of *Enterococcus cecorum* septicemia, which has been described as a presentation for this re-emerging pathogen (1). The age range of these birds at the time of submission is interesting as there are many submissions from this integrator for regular "sick chick" cases in which samples of yolk sac +/- bacterial lesions are sampled and aerobically cultured. None of these samples have come back as positive for *Enteroccocus cecorum* whereas Jung *et al*. (year) described the isolation of this organism in yolk sacs and spleens in a cross sectional study. This is not meaning that *Enterococcus cecorum* is not present in the bird at this age since it is described as a normal inhabitant of the GI tract (1). However, it can be concluded that it is not causing a septicemic infection or localized infection of the yolk sac at this age. This opens the question to where this infection is coming from. Currently the source of pathogenic *Enterococcus cecorum* is unknown (3). Vertical transmission has not been definitively proven (3). In addition, Borst. *et al*. (2016) were unable to isolate *E. cecorum* from the feed, water, litter, or dust in one study from houses experiencing or have experienced an outbreak of enterococcal spondylitis. However, we have been able to isolate *E*. *cecorum* from infraorbital sinuses in birds with swollen head from this integrator which have been submitted since this study took place, and in previous years this bacterium was isolated from litter samples (unpublished data). This is similar to Beata Dolka *et al*. (2017), who isolated *E. cecorum* from infraorbital sinuses of turkeys as well as trachea of laying hens. This further reiterates the cartilage and bone tropism that *E. cecorum* is reported to have as well as offer a potential route of infection for pathogenic strains in addition to the strains that are considered normal flora of the intestinal tract.

There were just three isolates from vertebrae/spine. These isolates were from birds showing clincial signs for vertebral osteoarthritis. This number could be falsly low due to perosonel not bringing in these symmetrically paralyzed birds from the field, and typical necropsy practices that do not routinely check the vertebrae of birds unless there are clinical signs indicating a need to. In addition, the majority of isolates were from bone (femoral head necrosis) and/or joints confirming that *Enterococcus cecorum* has predilection for cartliages and bone (4).

In terms of antimicrobial sensitivity results, one of the most important areas to consider are the antibiotics which are commonly used in poultry production. Some of these to note are penicillin-G, tetracycline, oxytetracycline, and Tylosin. When observing the antimicrobial sensitivities for these antibiotics, all except for penicillin-G have greater than 40% resistance. This is particularly interesting to note as this integrator is no antibiotics ever (NAE) and has been for approximatley the past 7 years. This eludes to the possibility that antimicrobial resistance is still present from the prior use of antibiotics in this integrator. Though *Enterococci* spp. have low affinity penicillin binding proteins as described by Jung *et al*., beta-lactams seemed to be efficacious in most isolates with low level resistance noted for amoxicillin and penicillin-G; penicillin-G appeared to be efficacious which is similar to other authors findings (5) . As noted by Jung *et al*. (2018), our analysis showed that the isolates were usually resistant to aminoglycosides with the exception of gentamicin.

There were an overwhelming number of multi-drug resistant isolates. It has yet to be determined whether multi-drug resistant isolates are more or less virulent (11).

(The full length article will be published in an upcoming edition of *Avian Diseases*, pending acceptance.)

REFERENCES

1. Stalker, Margaret J., et al. "Arthritis and Osteomyelitis Associated with Enterococcus Cecorum Infection in Broiler and Broiler Breeder Chickens in Ontario, Canada." *Journal of Veterinary Diagnostic Investigation*, vol. 22, no. 4, 2010, pp. 643–645., https://doi.org/10.1177/104063871002200426.

2. Jung A, Chen LR, Suyemoto MM, Barnes HJ, Borst LB. A Review of Enterococcus cecorum Infection in Poultry. Avian Dis. 2018 Sep;62(3):261-271. doi: 10.1637/11825-030618-Review.1. PMID: 30339512.

3. Borst, L. B., et al. "Pathogenesis of Enterococcal Spondylitis Caused by Enterococcus Cecorum in Broiler Chickens." *Veterinary Pathology*, vol. 54, no. 1, 2016, pp. 61–73., https://doi.org/10.1177/0300985816658098.

4. Dolka B, Chrobak-Chmiel D, Czopowicz M, Szeleszczuk P (2017) Characterization of pathogenic Enterococcus cecorum from different

poultry groups: Broiler chickens, layers, turkeys, and waterfowl. PLoS ONE 12(9): e0185199

5. Dolka, B., Chrobak-Chmiel, D., Makrai, L. *et al.* Phenotypic and genotypic characterization of *Enterococcus cecorum* strains associated with infections in poultry. *BMC Vet Res* 12**,** 129 (2016). https://doi.org/10.1186/s12917-016-0761-1

6. Swayne, David E., and Luke B Borst. "Streptococcus and Enterococcus." *Diseases of Poultry*, Wiley-Blackwell, Hoboken, NJ, 2020, pp. 1006–1010.

Site of isolation	Number
Hock Joint	53
Bone (femoral head) & tendon	40
Liver	34
Heart/Pericardium	30
Vertebrae	3
Bone marrow	2
Other (Air sac/Sternal bursa/spleen)	4

Table 1.1 Number of isolates by location.

7. "Sensititre SWIN Software System." Thermo Fisher , https://www.thermofisher.com/order/catalog/product/SW1202. Accessed 3 Feb. 2022.

8. Weinstein, Melvin P. *Performance Standards for Antimicrobial Susceptibility Testing*. 30th ed., Clinical and Laboratory Standards Institute, 2021. \

9. "Break Points Used for Susceptibility Testing of Enterococcus." USDA. https://www.ars.usda.gov/ARSUserFiles/60400520/NARMS/ABXEntero.pdf

10. WHO. WHONET: Windows-based database software for surveillance of antimicrobial resistance. Boston, MA. 2011. Available at: https://whonet.org/.

11. Martínez, José L, and Fernando Baquero. "Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance." *Clinical microbiology reviews* vol. 15,4 (2002): 647-79. doi:10.1128/CMR.15.4.647-679.2002

Table 2.1 Percentage of resistance, susceptibility, and intermediate as well as MIC 50/90 and MIC Range of isolates for each antibiotic.

Antibiotic	Breakpoints	Number	%R	%I	%S	MIC50	MIC90	MIC Range
Amoxicillin	S<=8 R>=16	81	11.1	0	88.9	0.25	16	0.16-32
Ceftiofur	S<=2 R>=4	81	16	0	84	0.5	4	0.25-8
Clindamycin	S<=4 R>=16	81	*33.3	0	66.7	4	8	0.4-8
Enrofloxacin	S<=0.5 R>=2	81	8.6	12.3	79	0.25	1	0.12-4
Erythromycin	S<=0.5 R>=8	81	30.9	11.1	58	0.125	8	0.12-8
Gentamicin	S<=4 R>=16	78	9	5.1	85.9	2	8	0.5-16
Neomycin	S<=8 R>=9	78	48.7	0	51.3	8	32	2-64
Oxytetracycline	S<=4 R>=8	81	55.6	0	44.4	8	16	0.25-16
Penicillin G	S<=8 R>=16	81	23.5	0	76.5	0.125	16	0.03-16
Spectinomycin	S<=8 R>=32	81	82.7	6.2	11.1	128	128	8-128
Streptomycin	S<=2 R>=4	78	82.1	0	0	256	G256	8-2048
Sulfadimethoxine	S<=256 R>=257	81	34.6	0	65.4	256	G256	0.256-530
Tetracycline	S<=4 R>=16	81	49.4	8.6	42	8	16	0.125-16
Tylosin	S<=4 R>=8	81	44.4	0	55.6	3	48	0.2-40

Table 2.2 Resistance profiles of isolates.

Resistance Profile	Number of Antibiotics in Profile	Number of isolates per profile	% of isolates per profile
STR	1	1	1.2
NEO TYL	2	1	1.2
SUD SPT	2	2	2.5
SUD SPT STR	3	1	1.2
OXY TCY TYL	3	1	1.2
OXY SPT TCY	3	1	1.2
NEO SPT STR	3	9	11.1
NEO OXY STR	3	1	1.2
OXY SPT TCY TYL	4	1	1.2
OXY SPT STR TCY	4	3	3.7
NEO SUD SPT STR	4	4	4.9
GEN NEO SPT STR	4	1	1.2
ERY NEO SPT STR	4	1	1.2
ENR SUD SPT STR	4	1	1.2
ENR OXY SPT TCY	4	1	1.2
ENR NEO SPT STR	4	1	1.2
CLI ERY STR TYL	4	1	1.2
CLI ERY SPT TYL	4	1	1.2
CLI ERY PEN TYL	4	1	1.2
TIO NEO SPT STR	4	1	1.2
OXY SPT STR TCY TYL	5	1	1.2
OXY SUD SPT STR TCY	5	2	2.5
NEO OXY SPT STR TCY	5	2	2.5
NEO OXY SUD SPT STR	5	1	1.2
ERY OXY SPT STR TCY	5	1	1.2
ERY GEN SPT STR TCY	5	1	1.2
ENR OXY SPT STR TCY	5	1	1.2
ENR NEO SUD SPT STR	5	1	1.2
TIO CLI ERY OXY TCY	5	1	1.2
NEO OXY SUD SPT STR TCY	6	3	3.7
GEN SUD SPT STR TCY TYL	6	1	1.2
CLI NEO SPT STR TCY TYL	6	1	1.2
CLI NEO OXY SPT STR TCY	6	1	1.2
CLI ERY NEO SPT STR TYL	6	1	1.2
TIO OXY SUD SPT STR TCY	6	1	1.2
ENR GEN NEO OXY SPT STR TCY	7	1	1.2
ENR ERY OXY SPT STR TCY TYL	7	1	1.2
CLI ERY OXY PEN STR TCY TYL	7	1	1.2
CLI ENR ERY OXY SPT STR TCY	7	1	1.2
TIO CLI ERY OXY PEN SPT TYL	7	1	1.2
GEN NEO OXY SUD SPT STR TCY TYL	8	1	1.2

ERY NEO OXY SUD SPT STR TCY TYL	8	1	1.2
ERY GEN OXY PEN SPT STR TCY TYL	8	1	1.2
CLI ERY OXY PEN PT STR TCY TYL	8	1	1.2
CLI ERY NEO OXY SPT STR TCY TYL	8	1	1.2
CLI ERY GEN PEN SUD SPT STR TYL	8	1	1.2
CLI ENR ERY OXY SPT STR TCY TYL	8	1	1.2
CLI ENR ERY OXY PEN SPT TCY TYL	8	2	2.5
TIO ERY GEN NEO SPT STR TCY TYL	8	2	2.5
AMX CLI ERY OXY PEN SPT TCY TYL	8	1	1.2
AMX TIO CLI ERY PEN SPT STR TYL	8	1	1.2
TIO CLI ERY OXY PEN SPT TCY TYL	8	1	1.2
ENR ERY OXY PEN SUD SPT STR TCY TYL	9	1	1.2
AMX TIO CLI ERY PEN SUD SPT STR TYL	9	1	1.2
TIO CLI ENR ERY NEO OXY SPT STR TCY TYL	9	1	1.2
AMX CLI ERY GEN OXY PEN SUD STR TCY TYL	9	1	1.2
AMX CLI ENR ERY OXY PEN SUD SPT TCY TYL	10	1	1.2
AMX CLI ERY GEN NEO OXY PEN SUD SPT STR TCY TYL	12	1	1.2
AMX TIO CLI ENR ERY OXY PEN SUD SPT STR TCY TYL	12	2	2.5
AMX TIO CLI ENR ERY NEO OXY PEN SUD SPT TCY TYL	12	1	1.2

ISOLATION OF *HISTOMONAS MELEAGRIDIS* AND *PENTATRICHOMONAS HOMINIS* IN AN ENTEROHEPATITIS OUTBREAK IN TURKEYS

V. Durairaj, D. Higuchi, S. Clark, R. Vander Veen, and T. Campi

Huvepharma Inc., Lincoln, Nebraska, USA

SUMMARY

A case of blackhead (enterohepatitis) was suspected in a nine-week-old tom turkey flock located in the Midwest, USA. Gross pathology lesions in ceca and liver led to a presumptive diagnosis of enterohepatitis (blackhead). Cecal samples were collected for diagnostic investigation. Cecal samples were also cultured in the lab and gene sequencing was conducted using species-specific primers for *Histomonas meleagridis* and *Tetratrichomonas gallinarum*. The gene targeted sequencing confirmed the presence of *H. meleagridis* and *Pentatrichomonas hominis*. To the best of our knowledge, this is the first report of isolation of *P. hominis* in turkeys and the impact of *P. hominis* on turkey gut health needs to be further investigated. Thus, field studies play a vital role in understanding the circulating pathogens in turkey flocks.

INTRODUCTION

Enterohepatitis, commonly called blackhead disease (histomoniasis, histomonosis), is a deadly disease in turkeys causing up to 100% mortality (1). Enterohepatitis is caused by a protozoal parasite, *Histomonas meleagridis*. Generally, a presumptive diagnosis of enterohepatitis is made based on gross lesions in ceca and liver (1). Similar ceca and liver lesions are also induced by *Tetratrichomonas gallinarum* (2). Thus, histopathology, PCR, isolation, and identification of the pathogen is needed to confirm the causative agent. Field investigation studies are important in identifying the pathogens circulating in turkey flocks.

CASE REPORT

In Fall 2019, a histomoniasis outbreak was reported in a tom turkey flock (n=9000 toms/barn) located in the Midwest, USA. Previous histomoniasis outbreaks were reported in the same barn. Increased mortality and morbidity were again reported in the affected barn. Clinical signs such as "sulfur-yellow feces" and distinctive lesions of enterohepatitis were observed in ceca and liver. Turkeys with severe clinical signs were necropsied and cecal samples were collected and shipped in modified Dwyer's media (3).

The cecal samples were incubated in tissue culture flasks anaerobically at 40°C for 2-3 days. *Histomonas meleagridis* was observed on microscopic examination of the culture. DNA was extracted and gene targeted sequencing against 18S ribosomal RNA of *H. meleagridis* (4) and *Tetratrichomonas gallinarum* (5) was performed.

DISCUSSION

A presumptive diagnosis of blackhead was made based on the clinical signs such as "sulfur-yellow feces" and distinctive lesions in ceca and liver. Gene targeted sequencing indicated that this isolate was 99.81% identical to an Austrian field isolate 5642-C405 and grouped with genotype-1 cluster of *H. meleagridis.* Gene targeted sequencing against *Tetratrichomonas gallinarum* revealed that this isolate was 99.39% identical to *Pentatrichomonas hominis* reported in cats, dogs, goats, boas and owls. At this point it is unclear if *P. hominis* originated from the affected flock or was transmitted from other species. To our knowledge, this is the first report of isolation of *P. hominis* from turkeys and indicates that the role of *P. hominis* in enteric diseases of turkeys should be further studied. Thus, cecal culture and genomic evaluation both play a vital role in understanding the circulating pathogens in the field.

REFERENCE

1. Hess M, McDougald LR. Histomoniasis. In: Swayne DE, editor. *Diseases of poultry*. 14th ed. Ames (IA): Wiley-Blackwell. p. 1223–1230; 2020.

2. Landman WJM, Gantois N, van Eck JHH, van der Heijden HMJF, Viscogliosi E. *Tetratrichomonas gallinarum* granuloma disease in a flock of free range layers. *Vet Q*. 39:153-160; 2019.

3. Hauck, R., P. L. Armstrong, and L. R. McDougald. *Histomonas meleagridis* (Protozoa: Trichomonadidae): analysis of growth requirements in vitro. *J Parasitol*. 96:1–7; 2010.

4. Bilic I, Jaskulska B, Souillard R, Liebhart D, and Hess M. Multi-locus typing of Histomonas meleagridis isolates demonstrates the existence of two different genotypes. *PLoS One*. 21:9 (3):e92438; 2014.

5. Hess M, Kolbe T, Grabensteiner E, Prosl H. Clonal cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and a *Blastocystis* sp. established through micromanipulation. *Parasitology*. 33:547-554; 2006.

SEROLOGICAL EVALUATION OF TURKEYS CHALLENGED WITH *HISTOMONAS MELEAGRIDIS*

V. Durairaj and R. Vander Veen

Huvepharma Inc., Lincoln, Nebraska, USA

SUMMARY

Blackhead disease (histomoniasis) is a devastating disease in turkeys caused by an anaerobic protozoal parasite, *Histomonas meleagridis*. *H. meleagridis* induces distinct lesions in ceca and liver. The diagnosis is made based on gross pathology, histology, culture and PCR. While several studies have been conducted to understand the pathogenesis of histomoniasis, serological studies are very limited. Serological parameters provide more insights towards understanding the pathogenesis of the disease. The main objective of these studies was to evaluate the serological parameters in turkeys challenged with *H. meleagridis.*

In the first study, blood samples were to be collected at 9 and 14 days post-challenge. In this study, 27 day-of-age poults were divided into three groups and two groups were challenged with $1x10^3$ wild-type *H. meleagridis*/ dose by intra-cloacal or cloacal-drop route, respectively. Fifty percent of the poults were necropsied 9 days post-challenge and blood samples were collected for serological investigation. Due to increased mortality among remaining poults, blood samples were not collected 14 days post-challenge. The serological evaluation revealed significant decrease in the serum cholesterol and alkaline phosphatase (ALKP) levels in turkeys challenged with *H. meleagridis*.

In second study, 15 day-of-age poults were divided into three groups and two groups were challenged with $1x10^4$ and $1x10^5$ wild-type *H. meleagridis* by intra-cloacal inoculation, respectively. Nine days post-challenge all the poults were necropsied and serum samples were analyzed. Poults challenged with *H. meleagridis* again had significantly decreased levels of serum cholesterol and alkaline phosphatase.

In both studies, depletion of serum cholesterol and alkaline phosphatase were noticed in turkeys challenged with *H. meleagridis.*

INTRODUCTION

Histomoniasis, commonly called blackhead disease, has a very high impact on the turkey industry due to high mortality and morbidity associated with it (1). High mortality up to 100% is noticed in turkeys within a few days to weeks from disease onset (1). It is well known that histomoniasis induces distinct lesions in ceca and liver (1). Several pathological and immunological studies have been conducted to understand the pathogenesis of histomoniasis. However, serological information from turkeys challenged with *H. meleagridis* would help in understanding this disease further. The objective of these studies was to evaluate if any serological imbalance was induced by *H.meleagridis* in turkeys.

MATERIALS AND METHODS

Study 1. Three groups of poults were enrolled in this study. Nine poults were enrolled in group 1 and served as negative controls. Ten poults were enrolled in both groups 2 and 3. All poults were raised in isolators and received *ad libitum* feed and water. At 27 days-of-age, poults in groups 2 and 3 were challenged with $1x10^3$ wild-type *H. meleagridis*/ dose by cloacal-drop and intra-cloacal route, respectively. Fifty percent of poults were necropsied 9-days post-challenge and blood samples were collected for serological investigation. Between 9-14 days, 5/5 poults (group 2) and 3/5 poults (group 3) were dead, thus no serum samples were collected from those poults.

Study 2. Three groups of poults were enrolled in this study. Four poults were enrolled in group 1 and served as negative controls. Fifteen poults were enrolled in both groups 2 and 3. All poults were raised in isolators and received *ad libitum* feed and water. At 15 days-of-age, poults in groups 2 and 3 were challenged $1x10^4$ and $1x10^5$ wild-type *H.meleagridis*/ dose, respectively. Nine days post-challenge blood samples were collected for serological investigation.

RESULTS

Study 1. The geometric mean of the alkaline phosphatase (ALKP) level in group 1 negative controls was 4693.93±1171.70 (U/L) while ALKP levels in groups 2 and 3 were 2593.44±585.10 (U/L) and 1551.79±529.70 (U/L), respectively. The geometric mean of the cholesterol level in group 1 negative controls was 135.36±14.34 (mg/dL), while cholesterol levels in groups 2 and 3 were 74.37±13.92 (mg/dL) and 55.87±12.65 (mg/dL), respectively.

Study 2. The geometric mean of the alkaline phosphatase (ALKP) level in group 1 negative controls was 3085.18±356.22 (U/L) while ALKP levels in groups 2 and 3 were 1344.01±713.29 (U/L) and 782.58±404.74 (U/L), respectively. The geometric mean of the cholesterol level in group 1 negative controls was 194.31±22.08 (mg/dL), while cholesterol [levels] in groups 2 and 3 were 91.42±33.96 (mg/dL) and 59.28 ± 17.40 (mg/dL), respectively.

Several serological [parameters] were evaluated in both studies. Among the serological parameters evaluated, only ALKP and cholesterol levels were significantly declined in the challenged poults.

DISCUSSION

In study 1, ALKP and cholesterol were significantly decreased in groups 2 and 3. Turkeys challenged by the intra-cloacal route had significant depletion of ALKP and cholesterol compared to the cloacal drop challenge administration. Even though both challenge routes adversely affected the ALKP and cholesterol level in turkeys, the impact caused by intra-cloacal administration was comparatively more severe than the cloacal-drop route. In study 2, ALKP and cholesterol were again significantly decreased in groups 2 and 3. Turkeys challenged with $1x10^5$ *H. meleagridis*/dose resulted in lower levels of ALKP and cholesterol compared to turkeys challenged with $1x10^4$ *H. meleagridis*/dose. Thus, a dose dependent depletion of ALKP and cholesterol was noticed in the second study. These results demonstrate that challenge with *H. meleagridis* results in a reduction of cholesterol and alkaline phosphatase. Thus, serological evaluation has provided a valuable data in further understanding the pathogenesis of histomoniasis.

ACKNOWLEDGMENTS

We would like to thank Deborrah Higuchi, Laura Trygstad, Nerrisa Aherm, Jess Houchen, and April Cruse for their valuable contributions. We would like to extend our thanks to Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) for performing the serological assays.

REFERENCE

1. Hess M, McDougald LR. Histomoniasis. In: Swayne DE, editor. *Diseases of poultry*. 14th ed. Ames (IA): Wiley-Blackwell. p. 1223–1230; 2020.

RESULTS AND APPLICATIONS OF WHOLE GENOME SEQUENCING OF AVIAN MYCOPLASMAS

N. Ferguson-Noel, M. Ehsan, and M. Dos Santos

Poultry Diagnostic and Research Center, Athens GA 30605

SUMMARY

Avian *Mycoplasma* spp. are pathogens that continue to result in significant morbidity and mortality in poultry and considerable economic loss to the industry. Advances in the high throughput sequencing technology has made making sequencing full genomes more affordable but unfortunately, the genetic basis for virulence, transmission, host adaption etc., have not been completely determined for avian *Mycoplasma* spp. In this research several *M. gallisepticum* isolates were sequenced and analyzed in order to identify genome differences; included in the analysis were pathogenic isolates that were genetically-related to vaccine strains. Whole genome sequencing was performed using Illumina and the isolates compared to *M. gallisepticum* R_{low} reference genome and *M. gallisepticum* live-attenuated vaccine genomes. The collective contigs for each strain were annotated using fully annotated *Mycoplasma* reference genomes. The analysis revealed a wide spectrum of genetic differences among the isolates including presence and absence of genes with potential roles in virulence.

INTRODUCTION

Avian *Mycoplasma* spp. are pathogens that continue to result in significant morbidity and mortality in poultry and considerable economic loss to the industry. *Mycoplasma gallisepticum* control in the United States has primarily been based on the eradication of the organism, using negative replacement stock and biosecurity to maintain the mycoplasma-free status in breeders and their progeny. Live *M. gallisepticum* vaccines have been used for control in areas where the isolation of poultry flocks and eradication is not feasible. Live vaccines that are currently used to control MG include F strain (1, 2), 6/85 (3) and ts-11(4, 5).

Advances in the high throughput sequencing technology has made making sequencing full genomes more affordable but unfortunately, the genetic basis for virulence, transmission, host adaption etc., have not been completely determined for avian *Mycoplasma* spp. As mycoplasmas lack a cell wall and have limited metabolic options for replication and survival, these minimal bacteria have developed mechanisms for efficient colonization of the host and for escape from the host immune response. Compared to other pathogens, few virulence-related genes have been identified in *M. gallisepticum.* GapA, a primary cytadhesin, and CrmA, an accessory cytadhesin, mediate the attachment of this pathogen to the respiratory epithelium of the host (6). VlhA is a surface lipoprotein that undergoes phase variation; changing the bacterial surface architecture and allowing the mycoplasmas to escape immune surveillance (7, 8). *M. gallisepticum* has been shown to depend on the dihydrolipoamide dehydrogenase (Lpd), a component of the pyruvate dehydrogenase complex for host colonization and pathogenesis (9) and expression of MalF, an ABC transporter, has also been shown to be essential for persistence and involved in pathogenicity (10, 11).

In this research 44 *M. gallisepticum* isolates were sequenced and analyzed. These isolates consisted of vaccine-like isolates as well as "wild-type" field strains isolated from chickens and turkeys across the United States from 1984-2018. Publicly available genomes (n=33) were also included in comparisons.

MATERIALS AND METHODS

DNA extraction. *Mycoplasma gallisepticum* isolates were grown in Frey's modified broth and following color change, cells were centrifuged at 13,000 x g for 3 minutes, the supernatant discarded, and the cell pellets were reconstituted in 200 µl of phosphate buffered saline (pH=7). Genomic DNA was extracted using the QIAGEN DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, CA) following the manufacturer's recommendations.

Whole-genome sequencing, alignment and annotation. Whole genome libraries were produced using Illumina MiSeq (Illumina Inc.), following manufacturer's protocols. Raw sequence reads were

uploaded to PATRIC 3.5.43 (12) and genome assembly and annotation were performed. Sequence-based analysis of the annotated genomes, including variation analysis and proteome comparison, was accomplished using tools in PATRIC. The genomes were analyzed for unusual coding differences between the genomes (stops and frameshifts) relative to predicted proteins.

RESULTS

The genome size and content of the assembled genomes were typical of *M. gallisepticum*. Annotation of the genomes identified 723 - 970 protein coding sequences (CDS) including hypothetical proteins as well as proteins with functional assignments. Several potential virulence factors were identified among the genomes, including genes that are associated with lipid metabolism, DNA recombination and hypothetical proteins and lipoproteins. Other genes of interest that were identified included transporters and those involved in antibiotic resistance.

Some of the greatest variability seen among the *M. gallisepticum* strains was in the number of ***vlhA* genes** present and the DNA sequences of the *vlhA* genes. Approximately 40 – 53 *vlhA* genes were identified among the isolates. *M. gallisepticum* generally has several *vlhA* genes spread across five loci and making up about 10% of the genome (13). Antibiotic resistance associated genes that were present and showed variability among the genomes included DNA gyrase subunits *gyrA* and *gyrB* (fluroquinolones) and translation elongation factor Tu and G (tetracylines). In addition, multiple sequence comparisons of read files identified variations (SNPs, insertions, deletions) in the 16S rRNA and ribosomal proteins (tetracyclines) as well as the 23S rRNA (tylosin) of the isolates.

The presence (and number) of several enzymes involved in several metabolic pathways that would affect growth and survival of the *M. gallisepticum* in the host as well as *in vitro* varied among the isolates analyzed. This included likely differences in carbohydrate metabolism (pyruvate metabolism, galactose metabolism, pentose phosphate pathway, fructose and mannose metabolism, glycolysis/gluconeogenesis and the TCA cycle), lipid metabolism, lipopolysaccharide biosynthesis and amino acid metabolism (valine, leucine and isoleucine degradation, tryptophan metabolism, tyrosine metabolism).

Other enzymes that varied were involved in pathways associated with uptake of nutrients, most specifically iron acquisition (siderophore-mediated iron transport proteins) and generally various transporters (ABC transporters, ECF transporters). Several proteins that were identified as having variable presence among the isolated were predicted to have nuclease activity. Several mobile genetic elements (MGE) (likely transposons) were also predicted and the presence and location of these MGEs varied widely among the genomes.

Using phylogenetic analysis, the isolates could be divided into several clades the major ones including 6/85 vaccine and vaccine-like isolates, F-strain and F-strain like isolates, ts-11 and ts-11-like isolates and a house-finch isolate group. By conducting genome comparison analyses within a clade, genome differences associated with virulence and host adaptions could be more closely examined.

DISCUSSION AND CONCLUSION

One of the primary challenges in the development of improved avian *Mycoplasma* vaccines is that there are few genetic tools available to systematically introduce or identify genes or mutations in *Mycoplasma* that attenuates its virulence for its animal host. The precise nature of the attenuation in the available *M. gallisepticum* vaccine strains is not clear, however, with the growing number of bacterial genomes in the public domain, predictions can be made about how an organism behaves from its gene sequences.

The great variability seen in the number of *vlhA* genes has been observed previously (14). The *vlhA* genes encode immunodominant lipoproteins and hemagglutinins that undergo phase-variable expression. They are thought to be virulence determinants which facilitate establishment of chronic infection through immune evasion. The presence/absence or nonsynonmous mutations in genes predicted to produce proteins be involved in key metabolic pathways was also a key observation. These changes may affect the *Mycoplasma* cell viability by affecting nutrient uptake, structural integrity and flexibility in carbon consumption. As lipoproteins mediate pathogen-host cell interactions (signaling) allowing the pathogen to evade the host immune system and may also mediate cell adhesion to the host; disruption in these genes may indicate loss of, and/or functional shift away from, very specific transport or attachment functions affecting virulence.

Several sequences were predicted to be mobile genetic elements, primarily transposons, which adds further complexity to the interpretation of the data as

transposons may affect the coding sequence into which it is integrated but also neighboring genes. The impact of mobile genetic elements on bacterial genomes is multifold, including activation or repression of genes and DNA rearrangements resulting in deletions, inversions and gene amplification. Transposon-mediated genomic changes may alter virulence, host range, or tissue tropism.

It is important to note that the mutations identified that may be involved in attenuation were not the same in all of the attenuated (non-pathogenic strains). *M. gallisepticum* isolates vary widely in their relative pathogenicity and several factors including the host can influence the degree of pathogenicity observed (15). In general turkeys experience more severe clinical signs than those described for chickens (16). More research is necessary to clarify the likely role of these mutations in different host species.

In this research we identified the presence/absence and/or nonsynonymous mutations in genes predicted to produce proteins involved in key functions of *M. gallisepticum*. This framework will allow us to target specific genes for future work to confirm the effect of these genetic changes on the phenotype of an *M. gallisepticum* isolate.

REFERENCES

1. Luginbuhl RE, Tourtellotte ME, Frazier. MN. Mycoplasma gallisepticum - Control by immunization. *Annals of the New York Academy of Science.* 143:234-238; 1967.

2. Adler HE, McMartin DA, Shifrine M. Immunization against Mycoplasma infections of poultry. *American Journal of Veterinary Research.* 21:482-485; 1960.

3. Evans RD, Hafez YS. Evaluation of a Mycoplasma gallisepticum strain exhibiting reduced virulence for prevention and control of poultry mycoplasmosis. *Avian Diseases.* 36:197-201; 1992.

4. Whithear KG, Soeripto, Harrigan KE, Ghiocas. E. Immunogenicity of a temperature sensitive mutant Mycoplasma gallisepticum vaccine. *Aust Vet J.* 67:168-74; eng. 1990 May.

5. Whithear KG, Soeripto, Harringan KE, Ghiocas. E. Safety of temperature sensitive mutant Mycoplasma gallisepticum vaccine. *Aust Vet J.* 67:159-65; eng. 1990 May.

6. Indikova I, Much P, Stipkovits L, Siebert-Gulle K, Szostak MP, Rosengarten R, Citti C. Role of the GapA and CrmA cytadhesins of Mycoplasma gallisepticum in promoting virulence and host colonization. *Infect Immun.* 81:1618-24; 2013 May.

7. Tulman ER, Liao X, Szczepanek SM, Ley DH, Kutish GF, Geary SJ. Extensive variation in surface lipoprotein gene content and genomic changes associated with virulence during evolution of a novel North American house finch epizootic strain of Mycoplasma gallisepticum. *Microbiology.* 158:2073-88; 2012 Aug.

8. Szczepanek SM, Tulman ER, Gorton TS, Liao X, Lu Z, Zinski J, Aziz F, Frasca S, Jr., Kutish GF, Geary SJ. Comparative genomic analyses of attenuated strains of Mycoplasma gallisepticum. *Infect Immun.* 78:1760-71; eng. 2010 Apr.

9. Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca S, Jr., Geary SJ. Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (lpd), in Mycoplasma gallisepticum through in vivo screening of transposon mutants. *Infect Immun.* 74:931-9; 2006 Feb.

10. Tseng CW, Kanci A, Citti C, Rosengarten R, Chiu CJ, Chen ZH, Geary SJ, Browning GF, Markham PF. MalF is essential for persistence of Mycoplasma gallisepticum in vivo. *Microbiology.* 159:1459-70; 2013 Jul.

11. Mahdizadeh S, Masukagami Y, Tseng CW, Markham PF, De Souza DP, Nijagal B, Tull D, Tatarczuch L, Browning GF, Sansom FM. A Mycoplasma gallisepticum Glycerol ABC Transporter Involved in Pathogenicity. *Appl Environ Microbiol.* 87: 2021 May 11.

12. Wattam AR, Davis JJ, Assaf R, Boisvert S, Brettin T, Bun C, Conrad N, Dietrich EM, Disz T, Gabbard JL*, et al.* Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res.* 45:D535-D542; 2017 Jan 04.

13. Papazisi L, Gorton TS, Kutish G, Markham PF, Browning GF, Swartzell S, Madan A, Mahairas G, Geary SJ. The complete genome sequence of the avian pathogen Mycoplasma gallisepticum strain Rlow. *Microbiology.* 149:2307-2316; 2003.

14. Ricketts C, Pickler L, Maurer J, Ayyampalayam S, Garcia M, Ferguson-Noel NM. Identification of Strain-Specific Sequences That Distinguish a Mycoplasma gallisepticum Vaccine Strain from Field Isolates. *J Clin Microbiol.* 55:244-252; 2017 Jan.

15. Armour NK, Ferguson-Noel N. Evaluation of the egg transmission and pathogenicity of Mycoplasma gallisepticum isolates genotyped as ts-11. *Avian Pathol.* 44:296-304; 2015 Apr 30.

16. Armour NK. *Mycoplasma gallisepticum Infection*. 14 ed. Hoboken, NJ: Wiley-Blackwell; 2020.

IMMUNOPROPHYLAXIS AGAINST CLOSTRIDIAL CELLULITIS/DERMATITIS IN COMMERCIAL TURKEYS

A. Forga[A], D. Graham[A], K. Robbins[B], A. Smith[B], R. Senas Cuesta[A], M. Coles[A], C. Selby[A], G. Tellez-Isaias[A], C. Vuong[A], and B. Hargis[A]

[A]University of Arkansas, J. K. Skeeles Poultry Health Laboratory, Fayetteville, AR 72701
[B]Butterball, LLC 307 Dodgen Pl, Ozark, AR 72949

SUMMARY

Clostridial cellulitis or dermatitis affects commercial turkey flocks, primarily as they approach market age. In the field, this disease has been effectively controlled with antibiotics, but alternatives to antibiotics are needed. Bacterin-toxoid vaccination programs have been shown to prevent clostridial diseases in other species, including humans. Results from previous field studies indicate that vaccination with an experimental whole-cell *Clostridium septicum* (CS) bacterin-toxoid oil emulsion vaccine reduced clostridial dermatitis-associated mortality and antibiotic usage for some commercial turkey flocks, but vaccination was not always efficacious (1). To improve vaccine efficacy, studies were conducted to optimize the antigenic component of the experimental vaccine and to determine the appropriate antigen to adjuvant ratio, route, and volume for vaccine administration. It was determined that larger volume vaccine doses produced higher serum antibody immune response regardless of antigen:adjuvant formulation ratio or route of injection. No significant differences (P>0.05) were found between formulation ratios or between the subcutaneous and tail head injection sites.

INTRODUCTION

CS is the primary causative agent associated with clostridial dermatitis in commercial turkeys (2). Mortality and plant condemnations result in large economic loss for producers (3). CS is a Gram-positive, anaerobic, spore-former. The spores are incredibly resilient and long lasting in the environment. When conditions are conducive for growth, the spores will germinate, proliferate and produce multiple toxins. One in particular, the hemolytic, pore-forming alpha-toxin is the predominant virulent component of CS (4). Antibiotics have been therapeutically used after onset of disease, but with the consumer push for antibiotic-free production, the turkey industry has no commercially available option to prevent clostridial dermatitis-related mortality in commercial turkeys (1, 5). Vaccination with whole-cell CS bacterin-toxoid has been shown to be immunologically efficacious and reduce clostridial dermatitis-related mortality in commercial turkeys (1, 5). The purpose of the present study was to optimize CS antigen titer and determine an appropriate vaccine formulation, dosage volume, and route of administration.

MATERIALS AND METHODS

CS isolates. Two CS isolates (CS2, CS3) used in these experiments were obtained from fluid/emphysematous lesions from Clostridial dermatitis-affected carcasses and confirmed as CS using commercial anaerobic isolation panels (2).

General antigen production and vaccine preparation. To produce the bacterin-toxoid antigen, CS isolates were grown overnight in tryptic soy broth (TSB) with sodium thioglycolate (0.5%) and inactivated at select times with formalin to achieve a final concentration of 0.25%. Cell concentration was confirmed microscopically and toxin production was confirmed via hemolytic red blood cell (RBC) assay to determine antigen titer (1). CS bacterin-toxoid was combined with water-in-oil Seppic Montanide 71 R VG adjuvant at a ratio of 30:70 antigen/adjuvant unless stated otherwise. The homogenization technique utilized in this experimental vaccine was performed as previously reported (1).

***In vitro* morphology and hemolytic activity (Exp 1).** In Exp 1, an attempt was made to optimize CS alpha-toxin production. A 50L co-culture of CS2 and CS3 was monitored for pH, cell morphology, growth phase, and hemolytic activity. To obtain the initial inoculum for batch fermentation, CS2 and CS3 were grown separately overnight in 2L cultures of TSB with sodium thioglycolate at 37°C. This 4L of turbid culture was used to seed 46L of TSB/thio media in a steam-in-place fermenter bringing the final

volume to 50L. Samples were collected periodically and subjected to microscopy for cell counts, gross morphological changes, and hemolytic RBC assays.

Antigen comparison (Exp 2). To compare serum antibody response to high or low hemolytic-titered antigen, separate 50L batches of CS were grown to a hemolytic titer ranging from 64 hemolytic units (HU) to 1024 HU. The antigen was combined with Seppic Montanide 71 R VG adjuvant, as previously described. Cultures were mixed during incubation, excluding group 5 antigen (128 HU Static), which was incubated statically. The experimental vaccine was administered at seven weeks-of-age at a 0.5mL dose, subcutaneously in the nape of the neck. Treatment groups (n=20/group) were as follows: 1) non-immunized control, 2) 128 HU, 3) 512 HU, 4) 1024 HU, 5) 128 HU Static, 6) Combined 1024 HU/64 HU antigens at 1:1 ratio, 7) Combined 64 HU/512 HU antigens at 5:1 ratio. Blood was collected from the brachial vein and serum was used to determine pre-immunization serum antibody levels to CS alpha-toxin, as measured by ELISA, and evaluated weekly up to 12 weeks-of-age (five weeks post-vaccination).

Vaccine formulation, volume, and route of administration (Exp 3). The vaccine was produced at two formulation ratios: 30% antigen to 70% adjuvant or 50% antigen to 50% adjuvant. These two formulations were administered in a low (0.5mL) or high (1mL) dose and via two injection routes: subcutaneously in the nape of the neck (SQ) or intramuscularly in the tail head. Treatment groups included: 1) non-immunized control; 2) SQ, 0.5mL dose, 30:70 ratio; 3) SQ, 1mL, 30:70; 4) SQ, 0.5mL, 50:50; 5) SQ, 1mL, 50:50; 6) tail head, 0.5mL, 30:70; 7) tail head, 1mL, 30:70; 8) tail head, 0.5mL, 50:50; and 9) tail head, 1mL, 50:50. The vaccine was administered at six weeks-of-age. Blood was collected from the brachial vein (n=20-21/group) and serum was used to determine pre-immunization serum antibody levels to CS alpha-toxin, as measured by ELISA, and evaluated weekly up to 12 weeks-of-age (six weeks post-vaccination).

RESULTS

In Exp 1, it was determined that toxin production *in vitro* correlated with log phase cell growth and the peak hemolytic titer of 1024 HU coincided with the stationary phase 6.25h post-inoculation (PI). Hemolytic titer decreased rapidly during cell death, falling to 512 HU at 7h PI, 128 HU at 24h PI, and 64 HU between 24h and 48h PI. In Exp 2, at 6 weeks post-vaccination, the 512 HU and combined 5:1 (64 HU:512 HU) antigen elicited significantly *(P*<0.05) higher serum antibody levels against the CS alpha-toxin than the higher titer 1024 HU antigen and the Combined 1:1 antigen (Figure 1). However, the 1024 HU and Combined 1:1 antigens did not produce significantly higher serum antibody titers than the non-immunized control. After comparing administration route, dose volume, and vaccine formulation in Exp 3, it was determined that the 1mL dose elicited a numerically higher serum antibody titer than the 0.5mL dose (Figure 2). Additionally, the SQ 50:50 group that received the 1mL dose elicited a significantly higher immune response than the 0.5mL counterpart, as measured by ELISA. There were no significant differences between antigen/adjuvant ratio or route of inoculation.

DISCUSSION

Although virulence of CS has been attributed to the CS alpha-toxin, hemolytic ability is not the only metric to consider when investigating antigens for potential vaccine use. Our findings show that when producing a CS bacterin-toxoid, cellular growth phase at the time of formalin inactivation can have a significant impact on humoral immune response. These results, in addition to previous studies (6), revealed that larger dose volumes of a CS bacterin-toxoid oil emulsion vaccine may increase serum antibody titers to the CS alpha-toxin. However, our investigation showed no significant differences between the subcutaneous and tail head vaccine administration route at either the 30:70 or 50:50 antigen/adjuvant ratios. This indicates that either vaccine administration route could be considered when implementing a vaccination program.

(The full-length article will be submitted to a relevant journal.**)**

REFERENCES

1. Graham BD, Robbins KM, Teague KD, Graham LE, Tellez-Isaias G, Hargis BM. Evaluation of the efficacy of a candidate turkey cellulitis/dermatitis oil emulsion vaccine on immune response and mortality under laboratory and commercial conditions. J. Appl. Poult. Res. 28:818–825; 2019

2. Tellez-Isaias G, Pumford NR, Morgan MJ, Wolfenden A., Hargis BM. Evidence for *Clostridium*

septicum as a primary cause of cellulitis in commercial turkeys. J. Vet. Diagn. Invest 21:374-377; 2009.

3. Clark S, Porter R, McComb B, Lipper R, Olson S, Nohner S, H.L. Shivaprasad HS. Clostridial cellulitis and cellulitis: an emerging disease of turkeys. Avian Dis 54:788-794; 2010.

4. Kennedy CL, Krejany EO, Young LF, O'Connor JR, Awad MM, Boyd RL, Emmins JJ, Lyras D, Rood JI. The alpha-toxin of *Clostridium septicum* is essential for virulence. Mol. Microbiol. 57:1357-1366; 2005

5. Thachil AJ, McComb B, Andersen MM, Shaw DP, Halvorson DA, Nagaraja KV. Role of *Clostridium perfringens* and *Clostridium septicum* in causing turkey cellulitis. Avian Dis., 54:795-801; 2010

6. Thachil AJ, McComb B, Kromm M, Nagaraja KV. Vaccination of turkeys with *Clostridium septicum* bacterin-toxoid: evaluation of protection against clostridial cellulitis. Avian Dis. 57:214-219; 2013

7. Brown MD, Stoll ML, Scasserra AE, Butcher GD. Detection of antibodies to *Mycoplasma gallisepticum* in egg yolk versus serum samples. J. Clin. Microbiol., 29:2901-2903; 1991

8. Davies RH, Heath PJ, Coxon SM, Sayers AR. Evaluation of the use of pooled serum, pooled muscle tissue fluid (meat juice) and pooled faeces for monitoring pig herds for *Salmonella.* J. Appl. Microbiol., 95:1016-1025; 2003

Figure 1. Exp 2. Average serum antibody titer against *Clostridium septicum* (CS) alpha-toxin in 12 weeks-of-age turkeys (n=20/group), 5 weeks post vaccination. Turkeys were inoculated subcutaneously with 0.5mL of experimental CS bacterin-toxoid vaccine containing low, mid, and high levels of formalin-inactivated CS alpha-toxin at 7-weeks-of-age. Antibody levels as measured by ELISA (S/P) are plotted as the mean ± standard error. The ELISA absorbance value obtained for the positive control, negative control, and experimental samples were used to calculate the sample to positive control ratio. Average S/P = (mean of treatment group – negative control) / (positive control – negative control) (1, 2, 7, 8). Abbreviations are as follows: HU = hemolytic unit titer of CS culture at time of formalin inactivation, Combined 1:1 = 1024 HU/64 HU antigen at 1:1 ratio, and Combined 5:1 = 64 HU/512 HU antigen at 5:1 ratio. [a-b]Differing letters signify significant ($P<0.05$) differences.

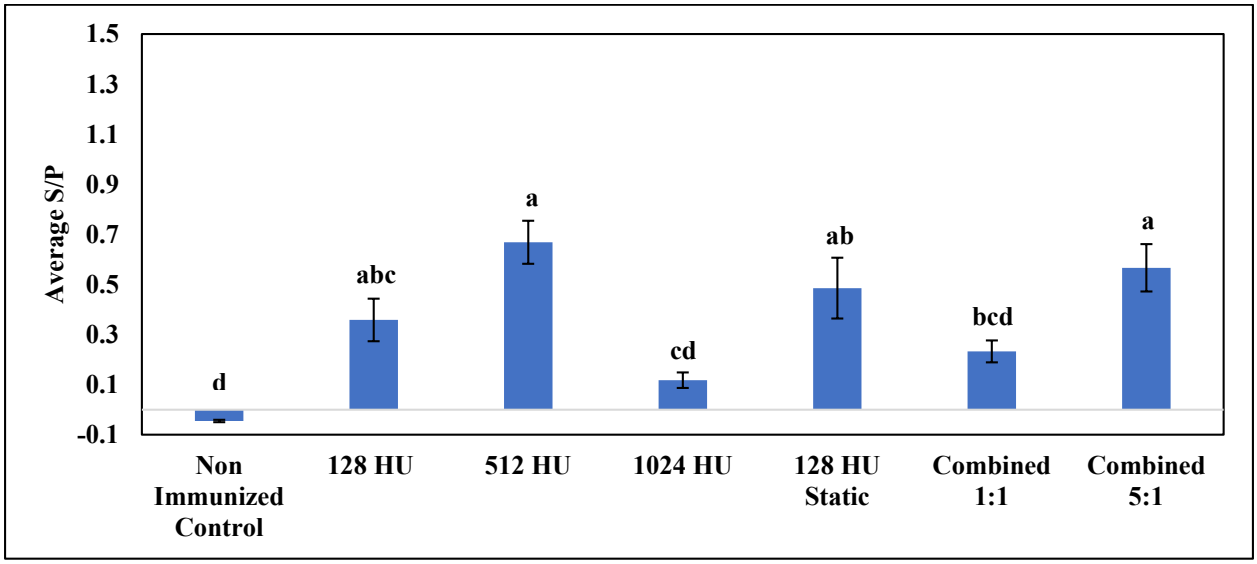
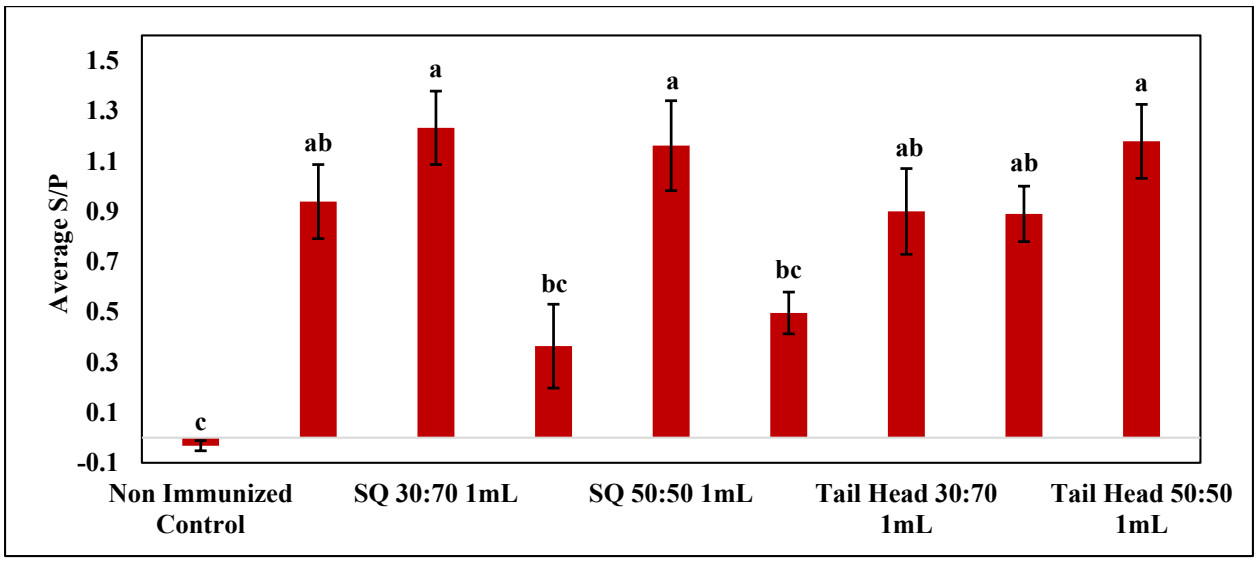


Figure 2. Exp 3. Average serum antibody titer against *Clostridium septicum* (CS) alpha-toxin in 12 weeks-of-age turkeys (n=20-21/group), 6 weeks post vaccination. Turkeys were inoculated subcutaneously (SQ) in the nape of the neck or intramuscularly in the tail head with 0.5mL or 1mL of experimental CS bacterin-toxoid vaccine formulated at 30:70 or 50:50 antigen/adjuvant ratio at 6-weeks-of-age. Antibody levels as measured by ELISA (S/P) are plotted as the mean ± standard error. The ELISA absorbance value obtained for the positive control, negative control, and experimental samples were used to calculate the sample to positive control ratio. Average S/P = (mean of treatment group – negative control) / (positive control – negative control) (1, 2, 7, 8). [a-b]Differing letters signify significant ($P<0.05$) differences.



INFLUENZA PATHOGENS: INNOVATIVE ASSAYS FOR DIAGNOSIS AND VACCINATION MONITORING OF H5, H7, AND H9 AVIAN INFLUENZA A VIRUS

M. Gaimard[A], S. Lesceu[A], C. Redal[A], J-E. Drus[A], C. Lefebvre[A], and P. Pourquier[A]

[A]IDvet, 310 rue Louis Pasteur, 34790 Grabels – FRANCE

SUMMARY

Influenza viruses belong to the family *Orthomyxoviridae* and infect a variety of human and animal hosts. There are four types of influenza viruses: A, B, C and D; which are defined by the nature of their internal nucleocapsid antigen. Most avian influenza viruses are low pathogenic, such as H9; but some subtypes, containing H5 and H7, are associated with highly pathogenic forms of the disease, with high rate of mortality. For disease diagnosis, competitive ELISAs are used for screening and serotyping. However, due to vaccination diffusion in countries, and given that competitive ELISAs are not suitable for monitoring of antibody, Innovative Diagnostics (IDvet) has developed unique indirect ELISAs to monitor vaccination uptake for H5, H7 or H9 AI. Vaccination monitoring with indirect quantitative ELISAs has the advantage to be highly correlated with HI test and could be used to monitor both conventional and recombinant vaccines (with DIVA strategies application for vector vaccines).

INTRODUCTION

Influenza viruses A, B, C and D, of the family *Orthomyxoviridae*, are responsible for influenza diseases affecting humans and certain animals. As to control outbreaks and limit the important losses in poultry flocks, vaccination with conventional or recombinant vaccines is more and more used. Given the need for rapid and reliable serological tools for monitoring of vaccination, Innovative Diagnostics (IDvet) has developed three indirect ELISA for H5, H7 and H9.

This poster presents validation data for the ID Screen® Influenza H5 and H9 Indirect for the monitoring of conventional or recombinant vaccines (data not shown for the ID Screen® Influenza H7).

MATERIALS AND METHODS

The ID Screen® Influenza H5 and H9 used respectively coated plates with H5 and H9 hemagglutinin recombinant proteins. Panels of known positive and negative samples, as described below, were tested as per manufacturer's instructions.

H9 iELISA. SPF chicken (origin: Hungary) were vaccinated with H9 recombinant vaccine (rHVT-H9, CEVA) at one day of age. Animals were challenged at 35 days of age with H9N2 virus. Antibody titers were evaluated using the ID Screen® Influenza H9 Indirect ELISA (cut-off: 732), the ID Screen® Influenza A Nucleoprotein Indirect (cut-off: 668) and the HI test (H9N2 Middle-East antigen, performed by CEVA Phylaxia). Animals were bled at 7, 14, 21, 28, 35, and 42 days.

Sera from commercial layers or broiler breeder flocks (origin: Jordan) were vaccinated with H9 inactivated vaccine at six weeks and 15 weeks of age (two vaccine program) or at 10 days, six weeks, and 15 weeks of age (three vaccine program). Antibody titers for each flock were evaluated using the ID Screen® Influenza H9 Indirect ELISA at five to eight weeks post-vaccination.

H5 iELISA. Chicken layers (origin: Hungary) were vaccinated with H5 recombinant vaccine (rHVT-H5, CEVA) at one day of age. Animals were challenged at 55 weeks of age with H5 homologous strain. Antibody titers were evaluated using the ID Screen® Influenza H5 Indirect ELISA (cut-off: 732), the ID Screen® Influenza A Nucleoprotein Indirect (cut-off: 668) and the HI test (performed by CEVA Phylaxia). Animals were bled at 55 weeks and 57 weeks (two weeks post-challenge).

For all studies, mean titers, minimum and maximum titers and the %CV were the parameters measured.

RESULTS

H9 iELISA. The H9 iELISA, based on a recombinant protein, demonstrated excellent sensitivity to detect antibody response to H9

recombinant and killed vaccine, in accordance with the HI test.

The ID Screen® Influenza A Nucleoprotein Indirect may be used as part of a DIVA strategy if the recombinant vaccine is used alone.

H5 iELISA. The H5 iELISA, based on a recombinant protein, demonstrated excellent sensitivity to detect antibody response to H5 recombinant vaccine.

DISCUSSION AND/OR CONCLUSION

The ID Screen® Influenza H5 and H9 Indirect kits are

- the only quantitative ELISAs for the specific detection of H5 or H9 antibodies
- the only test which allows H5 or H9 vaccination monitoring for conventional and recombinant vaccines
- able to detect field infection in vaccinated flocks

ACKNOWLEDGEMENTS

Innovative Diagnostics would like to thank CEVA Phylaxia, Hungary for the rHVT-H5 and H9 samples.

THE ROLE OF IBV ON REPRODUCTIVE IMPAIRMENT IN ROOSTERS

R. Gallardo[AE], A. da Silva[A], R. Gilbert[B], M. Alfonso[C], A. Conley[A], K. Jones[C], P. Stayer[B], and F. Hoerr[D]

[A]Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis. 1089 Veterinary Medicine Drive, 4008 VM3B, Davis, CA, 95616
[B]Sanderson Farms, Inc. 127 Flynt Road, Laurel, MS, 39443
[C]Ceva Animal Health. 8906 Rosehill Road, Lenexa, KS, 66215
[D]Veterinary Diagnostic Pathology, LLC. 638 South Fort Valley Road, Fort Valley, VA, 22652

ABSTRACT

Infectious bronchitis is a respiratory disease of chickens caused by a gammacoronavirus named infectious bronchitis virus (IBV). In addition to the respiratory tract, IBV may also induce urogenital infections, inducing nephropathogenic disease and false layer syndrome in laying hens while in males it has been associated with epididymal lithiasis and epididymitis. Early IBV infection / vaccination might be related with early inflammation inducing an initial damage to the tissue where the virus replicates disregarding of the IBV genotype. In clinical cases of impaired fertility in male broiler breeders we have been able to detect, through histopathology, lymphocytic epididymitis, epididymal lithiasis and orchitis. In addition, we have detected the agent through immune histochemistry and RT-qPCR. Finally, through radioimmunoassay we have found a significant reduction of testosterone in clinically affected compared with normal males. In conclusion, reproductive impairment is one of the consequences of IBV infection in male broiler breeders and while one particular strain might be associated all strains have the potential to cause disease in the reproductive tract. Additional factors such as management and nutrition should be considered and studied in these cases. In addition, continuous IBV surveillance to monitor vaccine strains and detect emerging variants is crucial to associate these clinical conditions with a specific variant strain and prevent it.

INTRODUCTION

The IBV-induced reproductive tract lesions in males are not as well described as in hens. It has been hypothesized that epidydimal stones are formed because of chronic inflammation in the efferent ducts, and that early IBV infection in males could be initiating these long-term inflammatory responses (1). This hypothesis was later confirmed in experiments using SPF male chickens vaccinated with live-attenuated and killed Mass-type IBV vaccines, where vaccinated birds developed epidydimal stones, atrophied testicles, reduced sperm production, and reduced serum testosterone levels compared with unvaccinated males (2, 3). The results seen in males vaccinated with a killed vaccine suggest that the stone formation is not induced by IBV replication in epithelial cells of the reproductive tract *per se*, but by the inflammatory and immunological responses that develop following IBV vaccination (3). In addition, epididymal stones have been detected in roosters without association to IBV and can be considered incidental findings that are not associated with subsequent reproductive pathology (4). Conversely, live viruses have been retrieved from testicular tissues from roosters presenting with low fertility, orchitis and epidydimal stones, suggesting a possible role of IBV replication in epidydimal stone formation (5).

Venereal transmission of IBV from males to females is also of concern, especially when young, sexually mature males are used to spike older hens to increase the flock's fertility. An experiment using mature laying hens artificially inseminated with IBV-spiked or naturally infected semen demonstrated that the virus could be detected in the tracheas of the hens and that their eggs were internally and externally affected by the IBV-infected semen, even though no histologic lesions were seen in the male testicular tissues (6).

In the last decade, the US broiler breeder industry has reported a significant decrease in hatchability (7), which is an indirect measurement of overall reduction in reproductive efficiency. This reproductive impairment could be partially explained by broiler breeder males affected by IBV.

MATERIALS AND METHODS

Histopathology. Sections of testes were collected in 10% neutral-buffered formalin and routinely processed for histology. Slides were stained with hematoxylin and eosin (H&E) and examined with a light microscope.

IBV immunohistochemistry. Unstained paraffin-embedded sections of testes were deparaffinized and used in an immunohistochemistry (IHC) assay using monoclonal antibodies against the spike (S) and nucleocapsid (N) proteins of IBV (8).

Testosterone quantification. Testosterone levels were measured using radioimmunoassay (9). Normal concentrations in roosters range from 1-10 ng/mL (2). Differences in testosterone concentrations between affected and unaffected flocks were assessed using an unpaired t test. Statistical differences were considered when $P<0.05$.

RESULTS

Histopathology. Testicles from affected males showed orchitis, characterized by interstitial lymphoplasmacytic infiltration (Fig. 1A). Most of the testicular lesions occurred in the epididymides, with moderate to severe necrosis of ductal epithelial cells and accumulation of cell debris and exudate within epidydimal ducts (Fig. 1B). Epididymal lithiasis was commonly observed as one or more calculi within connecting and efferent ducts and surrounded by proteinaceous casts, cell debris, and spermatozoa. Lymphocytic aggregates were present in the interstitium (Fig. 1C). Seminiferous tubules had sperm retention, and degeneration and necrosis of spermatids, resulting in extensive intratubular fibrinous to caseous accumulation.

IBV immunohistochemistry. Strongly positive IBV antigen staining was observed multifocally within the cytoplasm of sloughed epithelial cells and mononuclear inflammatory cells in testicles from 43% of the males that presented with gross and microscopic testicular lesions. The IBV IHC staining was mostly within efferent and connecting ducts where epididymal lithiasis was present (Fig. 1D).

Testosterone quantification. A significant difference was observed in testosterone concentrations between unaffected and clinically affected males (Fig. 2). The average testosterone level in unaffected roosters was 1.93 ng/mL, ranging from 0.72 to 5 ng/mL. The mean testosterone concentration was markedly lower in affected roosters, with an average of 0.56 ng/mL and within a range of 0.17 to 1.3 ng/mL.

DISCUSSION

IBV has been associated with reproductive problems in layers and breeders, especially when infections occur at an early age, causing oviduct dysfunction and false layer syndrome (10-13). Nevertheless, the role of IBV in male fertility is also a topic of increasing interest and importance.

The gross and histopathologic lesions observed in the affected roosters from this study are similar to those described in males that have been vaccinated with live or inactivated IBV vaccines or challenged with wild-type Mass strains (2, 3, 14, 15). While *in situ* detection of IBV in sections of testes presenting with microscopic lesions has been previously reported (5, 6, 16), this is the first time DMV/1639 has been associated with testicular pathology and reproductive impairment in males. In our study, IBV was detected by IHC in the cytoplasm of inflammatory and sloughed epithelial cells within epididymal ducts, amongst cell debris and calculi (Fig. 3D). However, IBV was only detected in three out of seven testicles showing lesions. It is likely that the IBV infection cleared from some birds at the time of collection, but the inflammatory response was still present. Moreover, it has been suggested that the IBV-related epididymitis and lithiasis observed in roosters with low fertility is likely related with the inflammation induced by the IBV infection or vaccination instead of *in situ* viral replication (3). Thus, IBV may not have to be in the testes to induce damaging inflammatory responses, and this pathogenesis should be further studied.

Exposure to IBV before sexual maturity is a risk factor for epidydimal stone formation at puberty (14). In addition to sperm, inflammatory cells, and cell debris, epidydimal stones are composed of calcium, and these stones vary in size and number as the roosters age (1, 14). It has been suggested that elevated concentrations of calcium and vitamin D_3 in rooster diets could be contributing factors for epidydimal stone formation (1). Since roosters and hens are usually fed the same ration, roosters may receive higher concentrations of calcium and vitamin D_3 than needed throughout their lives. Urolithiasis has been induced in pullets fed with layer ration and challenged with IBV (17) and in naturally occurring outbreaks of infectious bronchitis with calcium-related renal disease (18). Epididymal stone formation is a multifactorial issue, and these findings suggest that feed composition is an important factor when assessing reproductive health of broiler breeder males.

In addition to the testicular lesions and IBV presence in testes, serum testosterone levels in roosters from the affected flocks were significantly low, with only one out of seven presenting with testosterone concentrations above 1 ng/mL. Normal testosterone limits of 1-10 ng/mL have been reported in SPF birds (2), but normal broiler breeder males have been shown to range between 0.4 and 6.4 ng/mL depending on the age of the roosters. Males with testosterone levels below 1 ng/mL and gross and microscopic testicular lesions may be considered infertile (4). Similar results have been reported previously, where IBV vaccination was thought to induce seminiferous tubule atrophy, reduced testicular size, and consequently reduced serum testosterone concentrations, leading to male reproductive impairment (2). Moreover, 2 out of 7 roosters from the unaffected flock that served as a control also presented testosterone levels below 1 ng/mL. This finding is noteworthy because it is possible that the males from this supposedly healthy flock are subclinically affected by a similar issue, which would support the overall decreasing trend in hatchability within the broiler breeder industry in the past decade (Fig. 1).

The unilateral testicular dysfunction should not necessarily induce a lower testosterone level since compensatory hypertrophy and testosterone production by the less affected testicle is expected (19). In females, the left ovary and oviduct are affected by IBV (20), which is thought to be a consequence of the physiological lack of development of the right ovary in chickens. It is unknown if IBV has a predilection for the right or left gonad in males or females. The left testis reportedly secretes more testosterone (19) and therefore might have higher testicular tissue androgen concentrations if this were a factor in the establishment of infection in males. Nevertheless, the lower systemic testosterone concentrations suggest a lack of compensatory secretion expected to re-establish the equilibrium of the hypothalamic-pituitary-gonadal (HPG) axis. This might indicate disruption of hypothalamic-pituitary function, and not simply reflect the gonadal lesions *per se*. On the other hand, perhaps the testicle that seems unaffected grossly is not functioning properly and is unable to compensate despite increased hypothalamic-pituitary stimulation. These observations need further investigation to better understand the effect of unilateral gonadal disruption, and perhaps the HPG axis, induced by IBV in chickens.

In conclusion, the reproductive impairment observed in these broiler breeder flocks is likely a consequence of poor fertility in males, and IBV DMV/1639 is one of the possible causative agents of this condition. To further elucidate the role of DMV/1639 in rooster infertility, *in vivo* challenge experiments must be performed. Nevertheless, this study demonstrates the importance of performing molecular surveillance of IBV to monitor vaccine strains and to detect emerging variants that can potentially hinder production.

(A full version of this manuscript will be published in *Avian Diseases*.)

REFERENCES

1. Janssen SJ, Kirby JD, Hess RA, Rhoads M, Bunick D, Bailey KL, Parsons CM, Wang H, Bahr JM. Identification of epididymal stones in diverse rooster populations. *Poultry Science.* 79:568-574; 2000 2000/04/01/.

2. Boltz DA, Nakai M, Bahr JM. Avian Infectious Bronchitis Virus: A Possible Cause of Reduced Fertility in the Rooster. *Avian Diseases.* 48:909-915; 2004.

3. Boltz DA, Zimmerman CR, Nakai M, Bunick D, Scherba G, Bahr JM. Epididymal Stone Formation and Decreased Sperm Production in Roosters Vaccinated with a Killed Strain of Avian Infectious Bronchitis Virus. *Avian Diseases.* 50:594-598; 2006 12/1.

4. Sarabia Fragoso J, Pizarro Díaz M, Abad Moreno J, Casanovas Infesta P, Rodriguez-Bertos A, Barger K. Relationships Between Fertility and Some Parameters in Male Broiler Breeders (Body and Testicular Weight, Histology and Immunohistochemistry of Testes, Spermatogenesis and Hormonal Levels). *Reproduction in Domestic Animals.* 48:345-352; 2013.

5. Villarreal LY, Brandão PE, Chacón JL, Assayag MS, Maiorka PC, Raffi P, Saidenberg AB, Jones RC, Ferreira AJ. Orchitis in roosters with reduced fertility associated with avian infectious bronchitis virus and avian metapneumovirus infections. *Avian Dis.* 51:900-4; eng. 2007 Dec.

6. Gallardo RA, Hoerr FJ, Berry WD, van Santen VL, Toro H. Infectious bronchitis virus in testicles and venereal transmission. *Avian Dis.* 55:255-8; eng. 2011 Jun.

7. Stats A. *Live production reports*. https://agristats.com. 2009-2021.

8. França M, Woolcock PR, Yu M, Jackwood MW, Shivaprasad HL. Nephritis associated with infectious bronchitis virus Cal99 variant in game chickens. *Avian Dis.* 55:422-8; eng. 2011 Sep.

9. Stabenfeldt GH, Hughes JP, Kennedy PC, Meagher DM, Neely DP. Clinical findings, pathological changes and endocrinological secretory patterns in mares with ovarian tumours. *J Reprod Fertil Suppl.*277-85; eng. 1979.

10. Morin M-EB, Chénier S, Bergeron N. *La bronchite infectieuse aviaire au Québec*. Québec, Canada: [modified 04/25/2018; accessed 02/17/2021]. https://www.mapaq.gouv.qc.ca/SiteCollectionDocuments/Santeanimale/Bulletins/BulletinzoosanitaireIBV.pdf; 2018.

11. Parent E, Gagnon-Francoeur A, Lanthier B, Hébert G, Buczinski S, Boulianne M. Diagnostic Accuracy of Ultrasonography to Detect False Layers in a Commercial Laying Flock Infected by an Infectious Bronchitis Virus Delmarva Genotype Causing Cystic Oviducts. *Avian Dis.* 64:149-156; eng. 2020 Jun.

12. Grogan K, França M, Jordan B, Nicholds J. Case report: Clinical and histopathological findings in the first reported case of false layer syndrome in Georgia. 2020.

13. da Silva AP, Giroux C, Sellers HS, Mendoza-Reilley A, Stoute S, Gallardo RA. Characterization of an Infectious Bronchitis Virus Isolated from Commercial Layers Suffering from False Layer Syndrome. *Avian Diseases.* 65:373-380; 2021.

14. Jackson UH, Boltz DA, Nakai M, Scherba G, Bunick D, Bahr JM. Prepubertal Exposure to the Live Attenuated Avian Infectious Bronchitis Virus Induces Epididymal Stones in the Rooster after Puberty. *The Journal of Poultry Science.* 43:280-285; 2006.

15. Pereira NA, Alessi AC, Montassier HJ, Pereira RJG, Taniwaki SA, Botosso VF, Rui BR, Richtzenhain LJ. Gonadal pathogenicity of an infectious bronchitis virus strain from the Massachusetts genotype. *Braz J Microbiol.* 50:313-320; eng. 2019 Jan.

16. Cook JKA. Recovery of infectious bronchitis virus from eggs and chicks produced by experimentally inoculated hens. *Journal of Comparative Pathology.* 81:203-211; 1971 1971/04/01/.

17. Niznik RA, Wideman RF, Cowen BS, Kissell RE. Induction of Urolithiasis in Single Comb White Leghorn Pullets: Effect on Glomerular Number 1. *Poultry Science.* 64:1430-1437; 1985 1985/08/01/.

18. Cowen BS, Wideman RF, Braune MO, Owen RL. An Infectious Bronchitis Virus Isolated from Chickens Experiencing a Urolithiasis Outbreak. I. In vitro Characterization Studies. *Avian Diseases.* 31:878-883; 1987.

19. Lowry MM. Compensatory Testicular Adjustments Following the Injection of Small Quantities of Androgen into Unilaterally Castrated White Leghorn Cockerels1. *Poultry Science.* 37:1129-1136; 1958 1958/09/01/.

20. Benyeda Z, Szeredi L, Mató T, Süveges T, Balka G, Abonyi-Tóth Z, Rusvai M, Palya V. Comparative histopathology and immunohistochemistry of QX-like, Massachusetts and 793/B serotypes of infectious bronchitis virus infection in chickens. *J Comp Pathol.* 143:276-83; eng. 2010 Nov.

Figure 1. Microscopic lesions observed in testes of 33- to 38-week-old broiler breeder males with suspected low fertility. (A) Lymphoplasmacytic orchitis with moderate sperm retention (black arrows), degeneration and necrosis of seminiferous tubules with large amounts of intratubular exudate. Scale bar = 50 µM. (B) Extensive interstitial infiltration of lymphocytes with necrosis and central coagulum of necrotic cell debris within epididymal ducts. Scale bar = 50 µM. (C) Epididymitis characterized by moderate multifocal lymphocytic aggregates (arrows) and several efferent and connecting ducts with sperm and lithiasis (black circles). The inset shows an efferent duct containing a calculus surrounded by proteinaceous material, cell debris, and spermatozoa. Scale bars = 200 and 50 µM (inset). (D) Positive infectious bronchitis virus immunohistochemistry staining in the cytoplasm of sloughed epithelial cells and mononuclear inflammatory cells within an epidydimal duct. Scale bar = 50 µM.

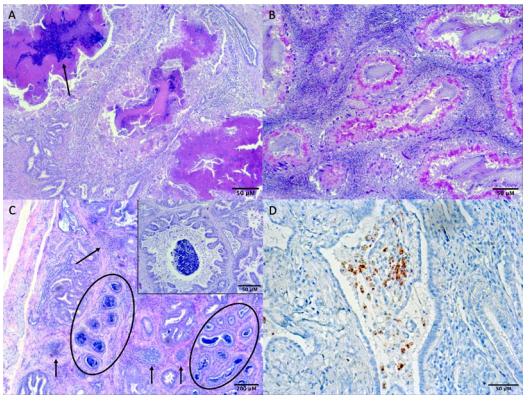
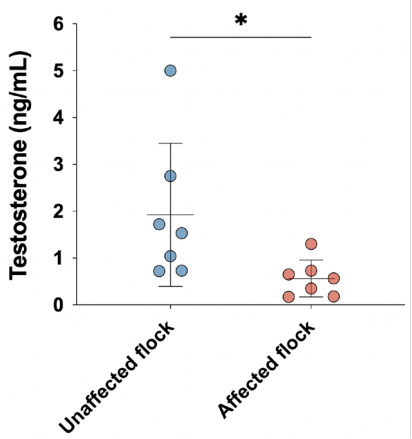


Figure 2. Testosterone levels in sera of 37-week-old broiler breeder roosters from a healthy flock (unaffected) and from a flock presenting with poor hatchability (affected). The asterisk represents a statistical difference between the groups (P<0.05). The whiskers represent the mean within one standard deviation.



DEVELOPMENT OF A NOVEL VACCINE STRATEGY AGAINST *CLOSTRIDIUM PERFRINGENS* BY SYNERGIZING IMMUNE-ENRICHMENT AND IMMUNOSTIMULATION IN CHICKS

H. Gautam[A], L. Ayalew[A], I. Subhasinghe[A], A. Shayeganmehr[A], S. Popowich[A], B. Chow-Lockerbie[A], K. Ashfaque Ahmed[A], and S. Gomis[A]

[A] Department of Veterinary Pathology Western College of Veterinary Medicine University of Saskatchewan 52 Campus Drive Saskatoon, SK S7N 5B4 CANADA

SUMMARY

The withdrawal of prophylactic antimicrobials use in the chicken industry has led to a substantial increase in various bacterial infections, including *Clostridium perfringens* (*C. perfringens*). In this study, we aimed at developing a novel vaccine strategy against *C. perfringens* by synergizing immune enrichment with immunostimulation in chicks. The experiment was conducted using four groups (n=35/group), 1- Controls, 2- *C. perfringens* challenge only, 3- In ovo CpG-ODN + inactivated *C. perfringens* vaccine, and 4- No CpG-ODN + Inactivated *C. perfringens* vaccine. The vaccinated birds received a booster dose at day 10 of their age. Groups 2, 3, and 4 were challenged with *C. perfringens* via feed (feed: media, 1:1) at days 20, 21, and 22, twice daily. Blood, intestinal mucosal scrapings, and tissues (for histopathology) were collected at the trial end. The serum IgY and intestinal mucosa IgA levels were observed significantly high in the vaccinated groups. Histopathological lesion scores revealed significantly high protection in group 3 compared to control group.

INTRODUCTION

Birds are generally most susceptible to infectious diseases at a young age, necessitating stringent management practices and vaccine strategies to control diseases and prevent economic losses in the poultry industry. In chickens, passively transferred maternal antibodies protect neonatal birds while the immune system matures. However, in broiler chickens, maternal antibody declines in about three weeks of age (1), rendering them vulnerable to pathogens. In a therapeutic antimicrobial-free chicken production scenario, immunosuppressive infections (such as Eimeria and IBDV) and lapses in managemental practices predispose broiler birds to *C. perfringens* infection that causes necrotic enteritis in chickens. It was estimated that necrotic enteritis inflicts economic losses costing around 6 billion dollars (USD) annually to the broiler chickens industry (2). These losses will increase amid the withdrawal of in-feed prophylactic antimicrobials in Canada. Therefore, there is an urgent need to find preventative strategies against necrotic enteritis. The objective of the study was to develop a novel vaccine strategy against *C. perfringens* by synergizing CpG-ODN-mediated immune enrichment with vaccinal immunity against *C. perfringens* antigen.

MATERIALS AND METHODS

Experiment design and bird care. This work was approved by the Animal Research Ethics Board, University of Saskatchewan, and adhered to the Canadian Council on Animal Care guidelines. Embryonated eggs (n=160) were incubated at the Animal Care Unit at the Western College of Veterinary Medicine, University of Saskatchewan, Canada. The eggs were candled at days 11 and 18 of incubation to confirm the viability. On day 18 of incubation, fertile eggs were randomly assigned into 4 groups (40 eggs each) 1- Controls, 2- *C. perfringens* challenge only, 3- *In ovo* CpG-ODN + inactivated *C. perfringens* vaccine, and 4- No CpG-ODN + Inactivated *C. perfringens* vaccine. The subsets of each vaccine group were made to determine the antibody response against each vaccine type at the trial end (n=10). Group numbers 3 and 4 were injected with 100 µL of CpG-ODN @ 50 µg/egg into the amniotic cavity using a 23G 1-inch needle. Melted wax was used to seal the injection site following *in ovo* CpG-ODN administration. At the day of hatch chicks in groups 3 and 4 were nebulized with lyophilized inactivated *C. perfringens* (10^8 cfu/ bird +CpG-ODN @10µg). Water and commercial broiler ration were provided ad libitum. Air from each room was exhausted through a high-efficiency particulate air

(HEPA) filter and non -recirculated intake air was provided at a rate of 15–20 air changes/hr. Air pressure differentials and strict sanitation were maintained in this isolation facility. Broilers were raised at 32° C for the first week of life. After that, the temperature was decreased to 0.5°C per day until a room temperature of 27.5° C was reached. The light was provided for 24 hr from days 0 to 2 post-hatch. Darkness was introduced at 3 days post-hatch with 1 hr of dark added daily until 4 hr of darkness was achieved. All the groups except groups 1 and 2 were boosted with inactivated *C. perfringens* (10^8cfu/bird) by the SC (Sub Cutaneous) route. All the groups except group 1 were challenged in feed with *C. perfringens* (1:1 ratio of $1x10^9$cfu/g feed) at 20, 21 and 22 days of age.

Sample collection. On day 24, blood samples were collected from ten birds per group in non-heparinized tubes and serum was separated. Serum samples were stored at -80°C until detection of IgY by our in-house conventional ELISA. The jejunal scrapings (n=10/group) were collected in 500µl of 0.1% EDTA and kept on ice for 10-15 minutes. The jejunal contents were vortexed for a few seconds and centrifuged @ 15000 rpm for 2 minutes. The supernatant was stored at -80°C for IgA detection by our developed in-house indirect ELISA. The duodenum, jejunum, and ileum samples were collected in 10% neutral buffered formalin for histopathology.

Antigen preparation. *C. perfringens* was cultured in 50mL of Fluid Thioglycollate medium for 16 hours in 100 mL capacity Erlenmeyer's flasks under anaerobic conditions (BD Gas Pak EZ chamber with 2 sachets of AnaeroGen 3.5L, Thermo scientific) at 37°C. The following day, 0.04% formaldehyde was added to the culture and incubated at 37°C for overnight. To ensure inactivation of *C. perfringens* 100 µL of overnight culture was plated on blood agar in duplicate. The inactivated culture solution was dispensed in 50mL centrifuge tubes in 35mL aliquots and centrifuged (Sorvall Legend RT, Mandel) at 4,000 rpm for 20 minutes. Bacterial pellets were transferred to small vaccine vials with 500uL of 10% sucrose and stored at -80°C overnight. The following day lyophilization was performed in the lyophilizer for 12 hours.

***C. perfringens* challenge.** *C. perfringens* was grown in a cooked meat medium (Sigma -Aldrich) for 24 h at 37 °C under anaerobic conditions (BD Gas Pak EZ chamber with 2 sachets of AnaeroGen 3.5L, Thermo scientific). Cooked meat medium was added to Thioglycollate medium (Sigma-Aldrich) at 3% (v/v). Cooked meat medium culture was incubated anaerobically at 37 °C for 15 h. The bacterial growth at 15 h culture had approximately 1×10^9 cfu/mL. The fluid Thioglycollate medium-grown culture was then mixed with feed at a ratio of 1:1 (v/w) for groups 1 and 2. Feed was withdrawn prior to exposure of birds to *C. perfringens*. It was administered twice daily in feed for 3 consecutive days.

Development of an indirect ELISA to detect IgA and IgY against *C. perfringens*. The indirect ELISA was developed to detect serum (IgY) and mucosal (IgA) antibody responses against *C. perfringens* whole cell antigen. The test was optimized using checkerboard titrations of *C. perfringens* whole cell antigen. The OD values were determined for test samples after subtracting the negative control OD values. The samples tested as follows:

Ninety-six well microtiter plates were coated with heat-inactivated *C. perfringens* (0.5 OD) and kept at 4°C overnight. Coated wells were blocked by the addition of 100ul of 10% skim milk diluted in phosphate-buffered saline (pH-7.4) with incubation for 30 minutes at room temperature. Blocked plates were then washed four times with distilled water. Sera were tested at 1:100 dilutions and mucosal scrapings at 1:10 dilution, in duplicates. The serum and mucosal supernatant from each bird were separately diluted in 1% skim milk diluted in PBST (pH -7.4). Then plates were washed 6X with distilled water and then added 100 uL of goat anti-chicken IgA (Gene tex) (1:2000) and incubated 2 hrs at room temperature. Plates were washed 6X with distilled water and added 100uL of 3,3′,5,5′-Tetramethylbenzidine (TMB substrate, Molecular innovations, Cedarlane) and incubated for 30 minutes. Negative controls consisted of triplicate wells with all reagents except either antigen, or primary, or secondary antibody. The optical density value (OD) at 405 nm, was determined using SpectraMax Plus 340 PC Microplate Reader. OD values for each bird were calculated as the average of OD405 values from duplicate wells minus the average OD405 values of the negative controls.

Statistical analysis. Statistical analysis of histopathological lesion scores was performed in Graph Pad Prism 6 (95% of Confidence interval). Turkey's multiple comparison test was performed after one-way ANOVA to compare the difference between the groups.

RESULTS

There were no NE lesions observed in-group without vaccine and *C. perfringens* challenge (Control) throughout the trial length. The group non-

vaccinated but challenged with *C. perfringens* showed 60 percent histopathology and 5 percent gross NE lesions. In both vaccinated groups (3,4) 20 percent of birds had NE lesions. The statistical analysis for histopathological lesions showed a highly significant difference ($p<0.01$) between group *C. perfringens* challenge only and in vaccinated groups followed by *C. perfringens* challenge. There was a significant increase in specific IgY production against *C. perfringens* in vaccinated groups compared to the non -vaccinated groups. Similarly, the production of intestinal mucosal IgA was significantly higher in group 3 (*in*-ovo CpG-ODN given inactivated *C. perfringens*) than in controls.

CONCLUSIONS

Vaccination by *in-ovo* CpG-ODN with inactivated *C. perfringens* produced a measurable mucosal and systemic immune response with a significant reduction in NE lesions post-challenge. A new effective vaccination strategy was developed against NE in broiler chickens.

REFERENCES

1. Ulmer-Franco, A., G. Cherian, N. Quezada, G. Fasenko, and L. McMullen. Hatching egg and newly hatched chick yolk sac total IgY content at 3 broiler breeder flock ages. Poultry science 91: 758–764. 2012.

2. Wade, B., and A. Keyburn. The true cost of necrotic enteritis. World Poult 31: 16–7. 2015.

UNDERSTANDING SPATIO-TEMPORAL TRENDS OF NON-COMMERCIAL POULTRY SALES IN CALIFORNIA USING ONLINE CLASSIFIED ADVERTISEMENT DATA

J. Gendreau[A], G. Koziol[A], S. Stoute[B], S. Ramsubeik[B], and M. Pitesky[A]

[A]University of California School of Veterinary Medicine, 1089 Veterinary Medicine Dr. VM3B, Davis, CA 95616
[B]California Animal Health and Food Safety Laboratory (CAHFS), Turlock, CA

SUMMARY

Backyard poultry ownership in Southern California is common and even increasing in many urban communities. The close proximity of backyard flocks to each other and to commercial flocks coupled with the general lack of strong biosecurity and vaccination practices facilitates the spread of infectious diseases, evidenced by the 2002 and 2018 outbreaks of virulent Newcastle disease (vND). While feed stores and mail order remain popular methods for purchasing chicks/hatching eggs, a large number of backyard birds and game fowl are also posted for sale on classified advertisement sites such as Craigslist.com. In order to gain a better spatio-temporal understanding associated with this type of on-line sales, a novel data mining software was developed to collect and categorize listings for backyard poultry sales from Craigslist.com subdomains across Southern California. Data collected using this tool can be used to better understand spatio-temporal trends in non-commercial poultry which can be used to offer insights related to disease modeling and extension.

INTRODUCTION

For many, keeping backyard poultry (BYP) and gamefowl (GF) is an engrained aspect of life in Southern California as both a food source and for recreation (1, 2). Unfortunately, in some cases, non-commercial poultry have been linked to the transmission of infectious diseases, including Newcastle disease in 2003 (3) and 2018 and *Salmonella* Hadar and *Salmonella* Enteritidis in 2021 (4) that resulted in over 200 human hospitalizations. With the increased ability to easily purchase poultry domestically and internationally coupled with the anecdotal apparent increase in BYP ownership over the course of the COVID-19 pandemic, the potential for future outbreaks is likely. Traditional survey methods are costly, time intensive and heavily biased. This is especially true when engaging with GF owners. The increased frequency and distance that GF are transported combined with the large number of other birds that GF interact with at exhibitions and, in some cases, fights increases the risk of disease transmission in these flocks. Additionally, GF are often shipped across state lines and internationally. In the case of a foreign animal disease outbreak, tracing the origin of these birds poses a challenge. Hence, more robust methods of monitoring spatio-temporal trends in BYP and GF ownership and practices are necessary. Several online classified advertisement sites including ebay.com, Facebook Marketplace, and Craigslist.com (CL) host hundreds of listings for BYP and GF that are updated frequently. CL is unique among these platforms in its popularity in Southern California and since transactions are made in person as opposed to shipped, hence limiting the distance birds are transported. Here we investigate the ability to use posting related to BYP and GF poultry on the on-line classified website Craigslist.com (CL) as a proxy for BYP ownership in a municipality or census designated place (CDP).

MATERIALS AND METHODS

Data collection. Craigslist.com "Farm + Garden" classified advertisement sections were scanned for listing for birds during two collection periods: the Initial Collection Period (ICP) from December 21st 2020 to January 21st 2021 and the Second Collection Period (SCP) from November 9th 2021 to January 14th 2022 (Second Collection Period). The Los Angeles, San Diego, Inland Empire and Imperial Craigslist subdomain (in "inlandempire.craigslist.com", "inlandempire" is the subdomain) Farm + Garden sections were scanned. Specifically, the classifieds were scanned once per day during the Initial Collection Period and once per week during the Second Collection Period. A computer

program was written in the C# language (5) using the .NET framework to process hypertext transfer protocol (HTTP) requests to the target sites the hypertext markup language (HTML) and cascading style sheets (CSS) documents. ScrapySharp (6) was used to emulate a web browser and target HTML tags and CSS selectors to retrieve target information.

On the main Farm + Garden pages, 120 listings per page are shown out of a total possible 3,000 listings per subdomain Farm + Garden section. The program opened these pages starting with the most recent post, and the hyperlink "<a>" tag text fields that contain the post title and page hyperlink for each listing were searched for the following criteria:

> **Match Terms**: "chicken", "rooster", "hen", "bird", "pigeon", "egg", "quail", "duck", "goose", "chick", "pullet", "gallos", "gallina", "huevo", "pato", "pavo", "guajolote", "codorniz", "turkey", "aseel", "asil", "scandaroon", "rhode"
>
> **Ignore Terms:** "cage", "wire", "coop", "trailer"

If the title text field contained words that matched any of the regular expression (regex) patterns under "Match Terms" and none of the regex patterns under "Ignore Terms", the hyperlink was added to a buffer. Once all listings on a page were scanned, the hyperlink to the next page was extracted. Then, the program "visited" each page in the hyperlink buffer and extracted the description, post date, and location (as reported by the poster). The number of days since the post was created was calculated. The program then assigned posts the following topic classifiers based upon regex matches in the title or description: Egg {"egg", "fertilized", "dozen", "huevo"}; Chicken {"chicken", "rooster", "hen", "pullet", "gallina"}; Pigeon{"pigeon"}; Rooster {"rooster", "cockerel", "gallo"}; Other Species {"quail", "duck", "goose", "pavo", "pato", "guajolote", "turkey", "codorniz"}. This process repeats until all Farm + Garden post titles have been screened for matching terms.

Data cleaning. Data cleaning was performed using Python (7) and the Pandas library (8) v1.1.3. The title field for each post was manually checked for key terms that corresponded to irrelevant posts (e.g. "bird" followed by "seed" or "statue"). The key terms were added to a list, which was then used to create a regex pattern. The title fields of each post were checked for the key terms and removed. This process was repeated until no false positives remained in the dataset.

Data analysis. Data was exported to a Microsoft Excel file after cleaning. Descriptive statistics were performed in Excel 2016 (9). Mapping was performed in ArcGIS Pro (10) using the California Board of Equalization (CBE) 2022 dataset (11). The total number of unique posts for each municipality and census designated place were spatially joined with the CBE data to create a choropleth map.

RESULTS AND DISCUSSION

In total, 23,048 posts were collected during the ICP and 7,477 posts were collected during the SCP. After cleaning, the cleaned ICP dataset consisted of 2,158 unique posts and the cleaned SCP dataset consisted of 2,597 unique posts for a total of 5,115 unique posts. For the purposes of this study, a "unique post" is defined as a unique URL and location combination since some posts were left online for multiple days or weeks and were recorded multiple times. The similar number of posts collected during the ICP (30-day collection period, one collection per day) and the SCP (11-week collection period, once per week) suggest that a significant number of posts are created and removed in less than 7 days, and weekly data collection does not fully capture the posts on a given Farm + Garden section during a given week. Tracking the number of days a given post is available (Figure 1) supports this by showing 54% of posts were available for 7 days or less.

The Inland Empire subdomain, which covers both Riverside and San Bernardino Counties, had the most posts out of the monitored subdomains during both the ICP (813 post, 37.7%) and the SCP (1,213 posts, 41%). Despite this, the city with the most unique posts during both periods was La Mesa in San Diego County (165 posts during ICP, 209 posts during SCP) followed by Vista in San Diego County during the ICP (89 posts) and Perris in Riverside County during the SCP (127 posts). The San Diego subdomain had the largest number of advertisements mentioning eggs (819 post). The Inland Empire subdomain had the largest number of advertisements mentioning chickens (1,434 posts), roosters (1,074 posts), and other species, including ducks, geese, and quail (457 posts). The Los Angeles subdomain had the largest number of posts offering pigeons for sale (341 posts).

Mapping unique post volume by municipality or census designated place (Figure 2a.) for both the ICP and SCP allows for further insight into "hot spots" of sales activity in the monitored areas of Southern California. The map detail focusing on western Riverside and San Bernardino Counties (Figure 2b.) identifies two major clusters of listing locations: the area including Riverside, Norco, Jurupa Valley, Fontana and Rialto, and the area including Perris,

Menifee, Lake Elsinore, and Hemet. Fontana, Riverside and Perris all were listed as sales locations in 120 or more unique advertisements. In the map detail showing San Diego County, El Cajon and La Mesa form a cluster in the southern part of the county while Vista and Escondido form a cluster in the northern part of the county. While both the Inland Empire and San Diego subdomains had significant numbers of unique sales posts, the locations listed in the advertisements appear more concentrated in a smaller number of municipalities in San Diego County. Since a significant number of posts in San Diego County list places in unincorporated sections of the county as the sales location, collecting and stratifying the data by zip code may allow for more insight.

Advertisement post volume over time did not change significantly during the SCP (Figure 3), but there was a marked reduction in volume during the ICP during the week of December 27th 2020 (Figure 4). Further monitoring over multiple holiday seasons is needed to gain more insight about sales activities during holidays. Changes in the volume of posts advertising chickens and roosters appear to change at the same rate as the total volume of posts over the two sampling periods.

The classified advertisement data explored in this study has the potential to improve the way that we respond to epizootic events. Currently, the California Animal Health and Food Safety (CAHFS) laboratory plays a critical role in surveillance and early detection of epizootics in the state, and detection relies heavily on BYP owners submitting birds or clinical samples for disease detection. Understanding where centers of bird ownership are in California and continuously comparing this with submissions data from CAHFS allows us to identify communities that are underrepresented in submissions and provide outreach. Additionally, if a quarantine is in place during an outbreak, monitoring advertisements for bird sales can inform responders of potential breaches of quarantine and allow for targeted response. Since BYP and GF are routinely shipped outside of the state and country, expanding monitoring to other popular BYP and GF classified advertisement platforms like eBay may allow for insight into shipped interstate and international sales. Continuous monitoring combined with text analysis may also provide more insight into seasonal trends in sales of poultry and GF specifically. Since GF have played a significant role in past outbreaks of vND and collecting information on GF through traditional survey methods is challenging, data available on CL and other classified advertisement sites can fill gaps in our understanding.

(Full length article will be published in a peer-reviewed journal.)

REFERENCES

1. [USDA] United States Department of Agriculture. *Urban Chicken Ownership in Four U.S. Cities*. USDA– APHIS–VS [accessed 2022 January 27]. http://www.aphis.usda.gov/animal_health/nahms/poultry/downloads/poultry10/Poultry10_dr_Urban_Chicken_Four.pdf; 2011.

2. Elkhoraibi C, Blatchford RA, Pitesky ME, Mench JA. Backyard chickens in the United States: A survey of flock owners. *Poultry Science*. 93(11):2920-2931, 2014.

3. [USDA] United States Department of Agriculture. *Epidemiological analyses of virulent newcastle disease in backyard birds in California*. USDA-APHIS [accessed 2022 January 28]. http://www.aphis.usda.gov/animal_health/downloads/animal_diseases/ai/epi-analyses-vnd-in-backyard-birds-in-california-dec.pdf; 2018.

4. [CDC] Centers for Disease Control and Prevention. Salmonella Outbreaks Linked to Backyard Poultry: Investigation Details. [modified 2021 November 18, accessed 2022 January 28]. http://www.cdc.gov/salmonella/backyardpoultry-05-21/details.html; 2021.

5. Microsoft Corporation. .NET Framework Reference, version 5.0 targeting C# version 9.0. http://docs.microsoft.com/en-us/dotnet/csharp/; 2020.

6. Romain F. ScrapySharp Library Reference, version 3.0.0. http://github.com/rflechner/ScrapySharp; 2018.

7. Python Software Foundation. Python Language Reference, version 3.7.11. http://www.python.org; 2021.

8. Pandas Team. Pandas Library Reference, version 1.1.3. http://pandas.pydata.org/; 2020.

9. Microsoft Corporation. Microsoft Excel version 2016. 2016.

10. ESRI. ArcGIS Pro version 2.8.0. https://www.esri.com/en-us/arcgis/products/arcgis-pro/overview; 2021

11. California State Board of Equalization. *City and County Boundary Line Changes.* [GIS]. 2022.

Figure 1. Number of days that classified advertisement posts are available on CL from date of posting. 54% of posts are available for 7 days or less from the date of posting.

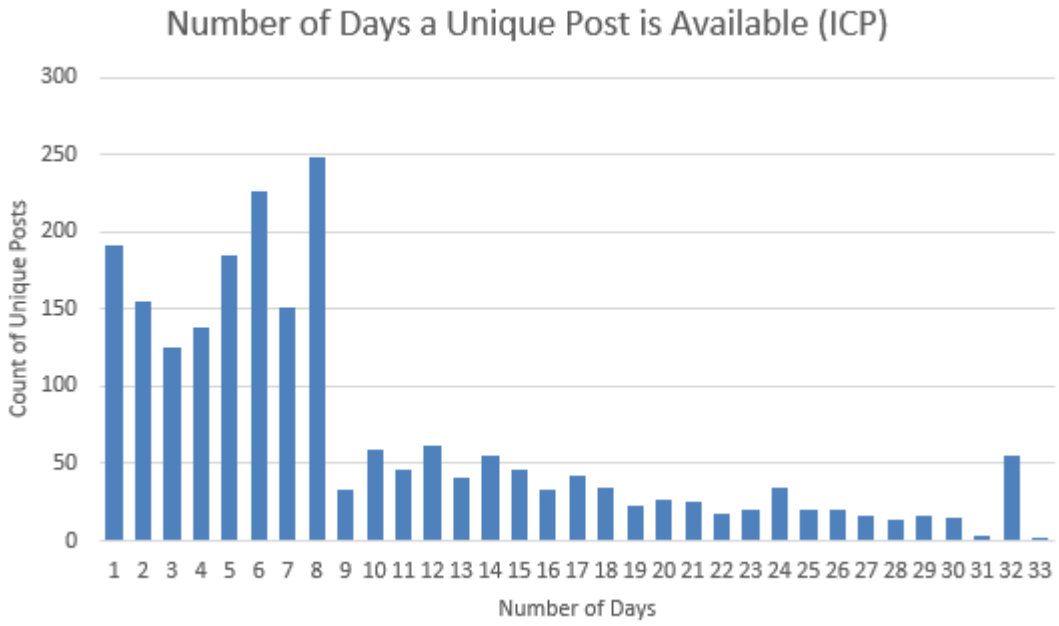


Figure 2a. Total number of unique CL classified advertisement posts during ICP and SCP mapped by municipality and census designated place (CDP). See Figure 2b. for western Inland Empire detail and Figure 2c. for San Diego County detail.

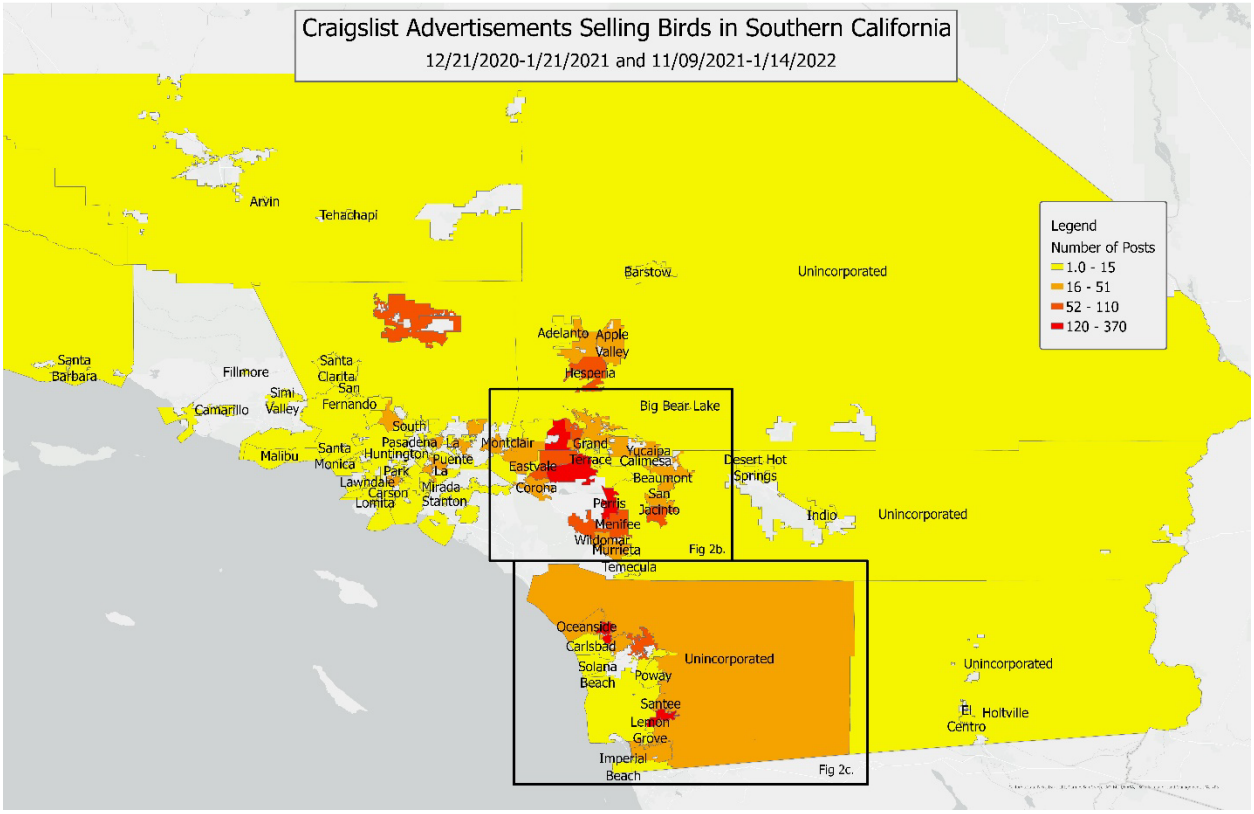


Figure 2b. Total number of unique CL classified advertisement posts during ICP and SCP mapped by municipality and census designated place (CDP) in western Riverside and San Bernardino Counties (Inland Empire).

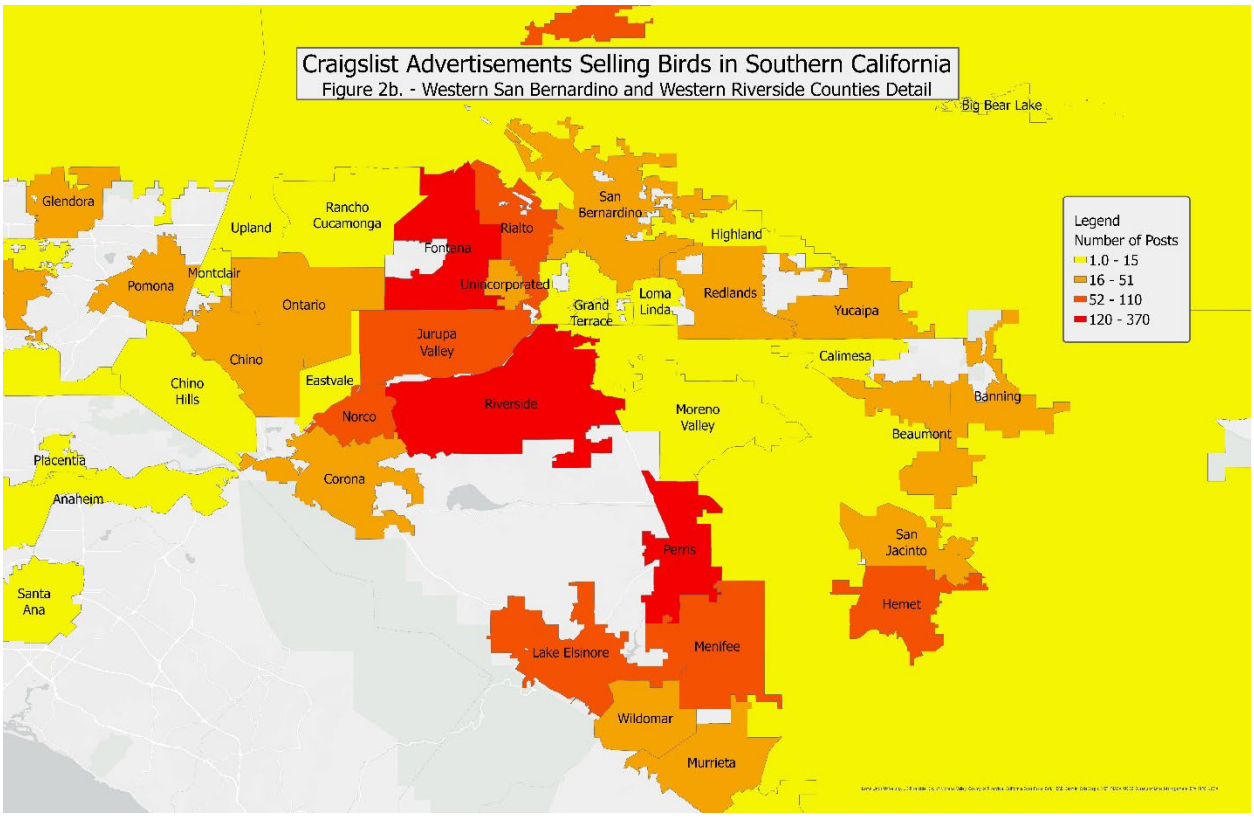


Figure 2c. Total number of unique CL classified advertisement posts during ICP and SCP mapped by municipality and census designated place (CDP) in San Diego County.

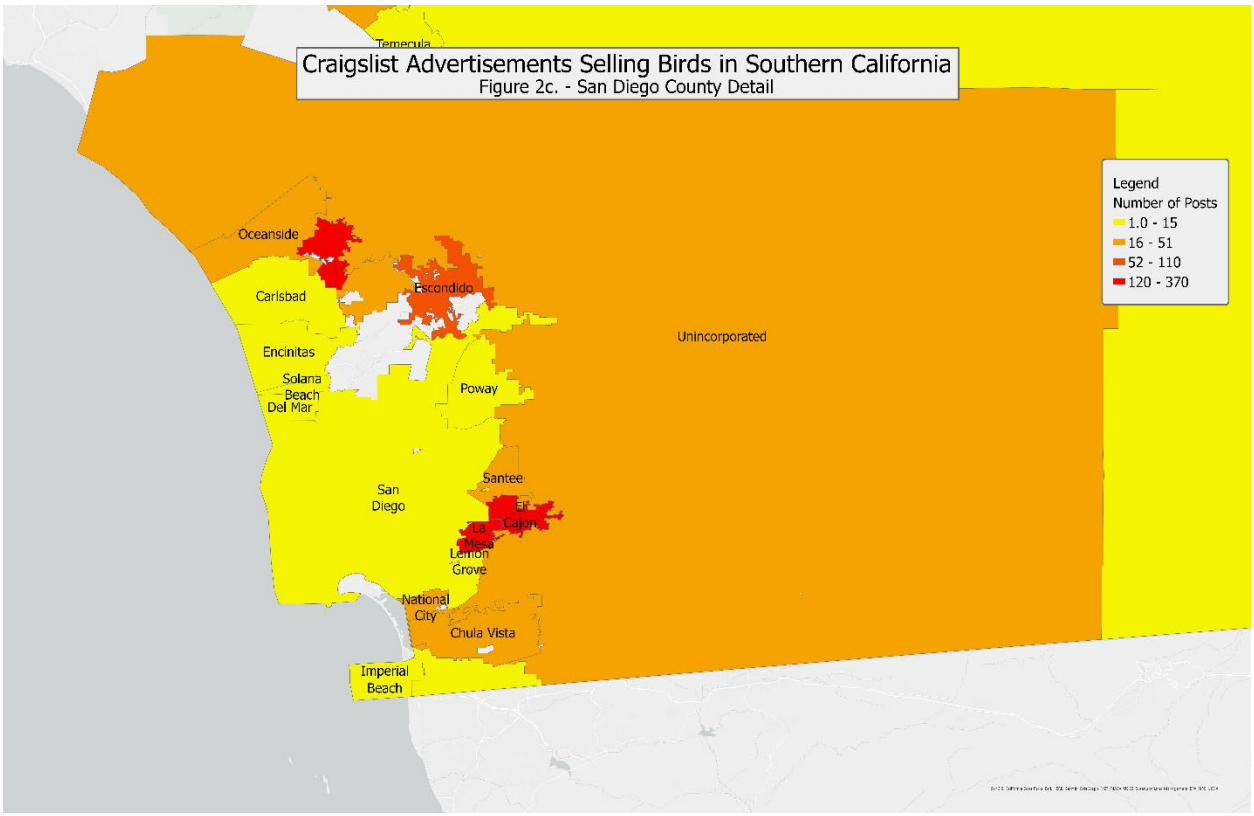


Figure 3. Volume of all CL posts selling birds, CL posts selling chickens, and CL posts selling roosters by week during SCP.

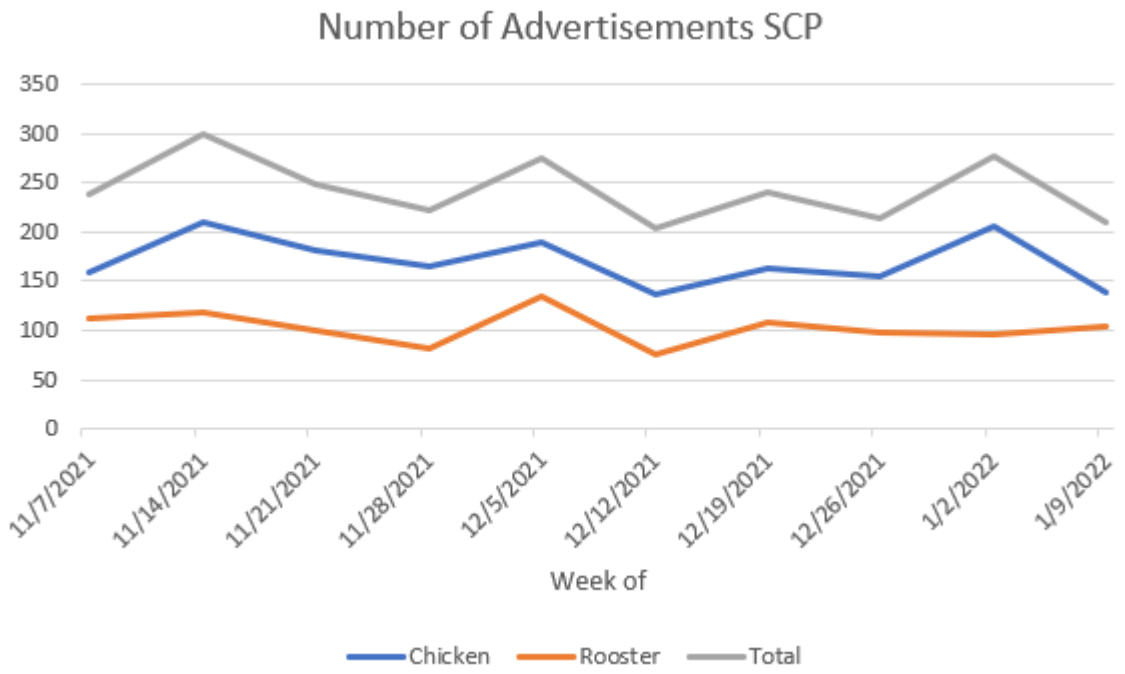
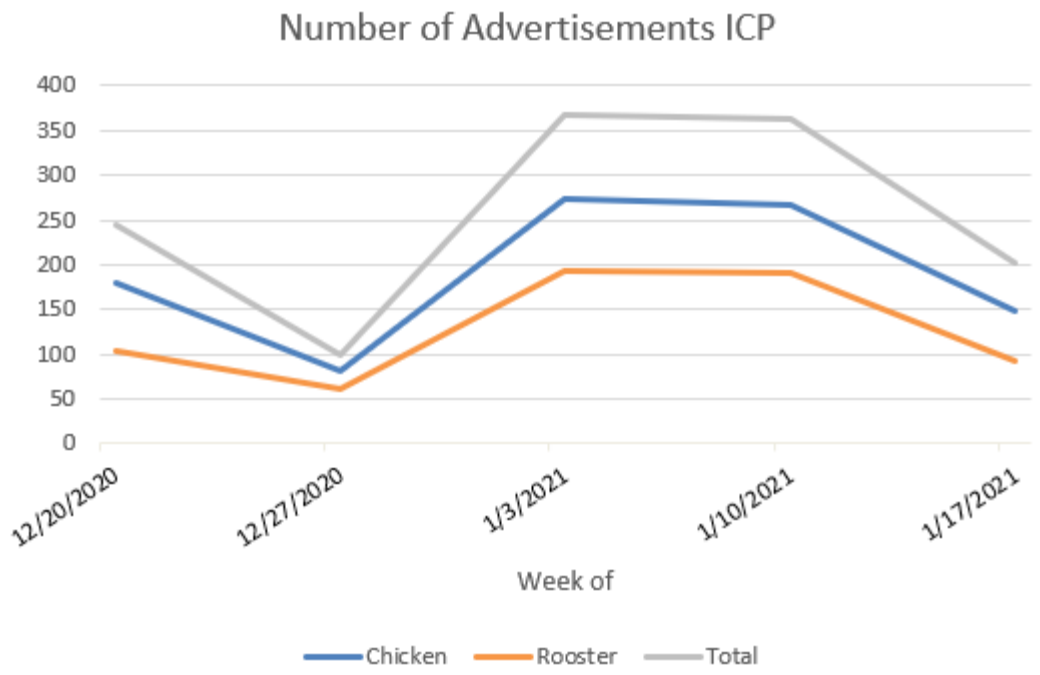


Figure 4. Volume of all CL posts selling birds, CL posts selling chickens, and CL posts selling roosters by week during ICP.



2021 OUTBREAK OF EGG DROP SYNDROME (EDS) IN NORTHERN INDIANA LAYER FLOCKS

E. Gingerich[A] and E. Blough[B]

[A]Diamond V, Zionsville IN 46077
[B]Crossroads Food Animal Veterinary Service, White Pigeon MI 49099

SUMMARY

An outbreak of egg drop syndrome (EDS) due to the adenovirus EDS '76 occurred on over 19 brown egg layer farms in northern Indiana starting in the fall of 2021 and continuing into 2022. The source of the infection is not known but suspected to be wild birds sharing pastures of outdoor access flocks. Affected flocks showed a significant reduction of sellable eggs, up to a 60% loss, due the production of very poor shell quality and shelless eggs. Mortality rates continued to be normal, and birds appeared healthy. Diagnosis was performed by the National Veterinary Services Laboratory in Ames IA by polymerase chain reaction (PCR) from shell gland, cloacal swabs, and swabs of shell membranes of shelless eggs. Most of the flocks were depopulated to reduce the number of infected flocks in the area and to reduce losses due to low production of sellable eggs. A reproductive rest was conducted in some flocks and resulted in bringing shell quality back to normal, but a substantial number of eggs were lost in the process. One flock that experienced the reproductive rest program was tested 10 weeks after initial signs and continued positive for virus in cloacal swabs, shell gland, and affected shells by PCR. Biosecurity practices are in the process of being upgraded to eliminate spread. Discussion of the possibility of the use of a killed vaccine in the area to improve control is ongoing.

INTRODUCTION

EDS is an adenoviral disease caused by the EDS '76 virus (EDSV), genus Atadenovirus, affecting mainly brown egg type layer chickens and resulting in an infection in the shell gland tissue. This infection results in the inability of the shell gland to put a normal shell on the egg with a shell membrane presented to it. Poor quality, thin shells with poor shell pigment and shelless eggs are the result. The ovulation rate of the hens is not affected but the percentage of marketable eggs is reduced dramatically, many times to 70% of normal. The virus reservoir in poultry producing areas is felt to be domestic poultry or wild waterfowl. No increase in mortality or morbidity is observed in affected flocks.

Diagnosis is conducted by submitting shell gland, cloacal swab, egg contents, and shell membrane swabs from shelless eggs in brain-heart infusion (BHI) broth with five swabs pooled per 5.5 mL. tube from at least 15 birds.

Prevention is by keeping the flock isolated from EDSV through biosecurity efforts. In most countries vaccination is a common practice using a single injected administration of an inactivated vaccine at 16 to 20 weeks of age. As the virus is non-enveloped, it is relatively resistant in the environment and difficult to eliminate from a premise. Non-vaccinated flocks placed in previously infected premises that were cleaned and disinfected have experienced outbreaks. The virus is susceptible to glutaraldehyde, formaldehyde, peroxygen, and chlorine dioxide disinfection but resistant to alcohols.

HISTORY

EDS is a rarely seen disease in the US. Only two premises in Indiana in 2011 (1) and five premises in Pennsylvania (2) since 2018 have been reported in the US prior to this outbreak. It may be a coincidence, but both northeast Indiana and southeast Pennsylvania where EDS has occurred most recently, are also the areas of highest duck production in the US.

The area of Indiana with the present outbreak, Elkhart and LaGrange counties, has grown greatly in the last 10 years to contain over 125 small layer farms, 3000 to 20,000 birds in a flock, producing specialty eggs, mostly brown eggs. This two-county area is a poultry dense area and is home to numerous other poultry; commercial meat duck, breeder duck, cage free white egg layer, cage free pullets, and broiler farms. Four of the five major companies with brown egg flocks in the two-county area have affected premises. The biggest nidus of infection occurred near Millersburg IN in a two-mile radius. One outlying flock that was 21 miles west of the Millersburg flocks

had the history of a service technician repairing egg belts seven days prior to clinical signs being seen. Most all the flocks had outdoor access at one time as most are organic flocks. Epizootiology interviews have been conducted by the Indiana State Board of Animal Health (BOAH) the results of which have yet to be released.

See Table 1. For the details of the flocks affected by EDS.

A confinement order was put in place in early November for any flocks that are either free range or organic that requires outdoor access. Flocks continued to break after the confinement order was issued.

The virus load was found to be highest from the samples of shell membrane of shelless eggs (testing done by NVSL by PCR). Shell membrane swabs were pre-moistened in fresh BHI broth prior to swabbing. Results of testing from one of the affected flocks one week after initial clinical signs is seen in Table 2.

A reproductive rest program using non-feed withdrawal was done in some flocks to restore egg quality back to normal using a relatively short process that kept the flock out of production for five to six weeks. One flock that went through the reproductive rest was tested for evidence of infection 10 weeks after initial clinical signs and was found to continue to be PCR positive with the results found in Table 3.

DISCUSSION

Despite improved biosecurity efforts including increased on farm usage of the Danish entry method, at the time of writing this document, the EDS outbreak is ongoing. Positive premises have been using great care to dispose of dead birds and cull eggs properly and utilizing additional cleaning and disinfection measures within the house. Disposal of litter from positive premises in addition to the depopulated carcasses of birds has created logistical challenges. Egg flats and pallets absolutely must be cleaned and disinfected, and care must be taken to ensure that improperly cleaned materials do not become distributed to farms. Positive farms are encouraged to utilize disposable PPE, gloves, and restrict access to birds and the eggs. EDSV is a relatively stable virus in the environment especially during winter months with low temperatures. The virus is also evidently not easily eliminated from a house after depopulation as a replacement flock from this outbreak became infected shortly after housing in a thoroughly cleaned and disinfected house. Egg belt sanitation is an especially difficult proposition due to much egg material being deposited from easily broken eggs.

Vaccination with a highly effective killed vaccine is being investigated with the state of Indiana and USDA if it is felt that biosecurity and depopulation efforts are not going to be able to control the spread of EDS within the area or into outlying areas.

REFERENCES

1. Personal communication, Dr. Richard Fulton, Michigan State University, 2011.

2. 2019 Midwest Poultry Federation Convention, EDS in Pennsylvania, Dr. Sherrill Davison, University of Pennsylvania.

Table 1. EDS Flocks in Northern IN 2021/2022 – Update of February 3, 2022, 1:02 pm.

Premise Designation	Company	Premise Location	Confirmed + by NVSL or Other Lab	Depopulated?	Outdoor Access	Initial Signs
A (1)	A	Millersburg	No	Yes	Yes*	30Aug21
B (2)	A	Millersburg	Positive	Yes 30Dec	Yes*	30Sep21-35w
C (3)	B	Millersburg	Positive	Yes, 19 Oct	Yes*	
D (4)	C	Millersburg	Positive, 18Oct21	Yes, 25 Oct	Yes*	04Oct21
E (5)	C	Millersburg	No	Yes, 25 Oct	Yes*	15Oct21
F (6)	A	Wakarusa	Positive	Yes, 18 Oct	No	10Oct21
G (7)	C	Millersburg	Positive, 25Oct21	Yes, 24Nov	Yes*	21Oct21-26w
H (8)	C	Millersburg	Positive, 08Nov21	Yes, 24Nov	Yes*	05Nov21-28w
I (9)	D	Millersburg	Positive	Yes, 22Nov	Yes*	06Nov21
J (10)	D	Millersburg	Positive	Yes, 23Nov	Yes*	13Nov21
K (11)	B	Topeka	Positive, 16Nov	Yes	Yes*	12Nov21

L (12)	A	Topeka	Positive	Yes, 23Nov	Yes*	13Nov21
M (13)	B	Millersburg	Positive	Yes, 24Nov	Yes*	18Nov21
N (14)	B	Goshen	Positive	No	Yes*	15Dec21-21w
O (15)	B	Shipshewana	Positive	No	Yes*	16Dec21-40w
P (16)	B	Shipshewana	Positive	Yes	Yes*	07Jan22-41w
Q (17)	C	Topeka	Yes	Yes	No	18Jan22-26w
R (18)	B	Shipshewana	Yes	Yes 26Jan	Yes	20Jan22 47w
S (19)**	A	Wakarusa	Yes	Yes	No	28Jan22 22w

* Outdoor access prior to confinement orders in mid-October
** Outbreak in a house that was previously infected following complete cleaning and disinfection.

Table 2. EDS PCR results of a recently infected flock.

Sample	PCR CT Value
Shell Membrane/Shelless Eggs	16.24
Cloacal Swab Pool A	24.87
Cloacal Swab Pool B	24.03
Cloacal Swab Pool C	22.39
Shell Gland Swab Pool A	32.96
Shell Gland Swab Pool B	28.12
Shell Gland Swab Pool C	33.29

Table 3. EDS PCR results of a "reproductive rested" flock, 10 weeks after initial clinical signs.

Sample*	**PCR Test**	**CT**
Cloacal 1	EDS	29.78
Cloacal 2	EDS	32.39
Cloacal 3	EDS	33.35
Cloacal 4	EDS	31.78
Cloacal 5	EDS	33.36
Cloacal 6	EDS	32.39
Shell gland 1	EDS	37.16
Shell gland 2	EDS	32.58
Shell gland 3	EDS	34.35
Shell gland 4	EDS	31.75
Shell gland 5	EDS	33.17
Shell gland 6	EDS	31.42
Intact poor shells 1	EDS	29.25
Intact poor shells 2	EDS	29.44

DEVELOPMENT OF A CANDIDATE BIOSHUTTLE COCCIDIOSIS VACCINATION PROGRAM FOR THE TURKEY INDUSTRY

D. Graham[A]*, C. Trujillo[A], R. Senas Cuesta[A], A. Forga[A], M. Coles[A], C. Selby[A], G. Tellez-Isaias[A], J. Barta[B], and B. Hargis[A]

[A] Department of Poultry Science, University of Arkansas Division of Agriculture, Fayetteville 72701, USA
[B] Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada

SUMMARY

Eimeria spp. that infect domestic turkeys have been recovered from wild turkey populations (1). As a result, we hypothesized that wild turkey feces collected from regions around the United States that were not in close proximity to commercial turkey operations would harbor drug-susceptible *Eimeria* spp. oocysts. Anticoccidial-sensitive *Eimeria* spp. strains isolated from wild turkey feces could be used to control coccidiosis in commercial turkey operations. To date, our group has successfully isolated 5/7 *Eimeria* spp. from wild turkey feces. The purpose of the present study was to evaluate the immunogenicity of a drug-sensitive, non-attenuated strain of *E. meleagrimitis* isolated from wild turkey feces. Vaccination, with and without amprolium treatment from day 10 to day 14, caused a very mild infection that induced protective immunity.

INTRODUCTION

Coccidiosis is caused by the genus *Eimeria.* These obligate intracellular protozoa invade and destroy host intestinal epithelial cells, and depending on the species and number of ingested sporulated oocysts, can significantly impact host health and production performance. Chemoprophylactics have been used for over a century to control coccidiosis in commercial poultry species (2). However, resistance to anticoccidial drugs in wild-type field strains has been observed to commercially available drugs (3). Due to the emergence and ubiquity of multidrug-resistant *Eimeria* spp., the turkey industry has limited options for coccidiosis prevention in commercial turkey flocks. Anticoccidial rotational and shuttle programs have extended to the use of some drugs, but live vaccination with drug-susceptible *Eimeria* spp. may displace drug-resistant wild-type *Eimeria* strains in the barn environment (4). A bioshuttle program (i.e. application of live coccidiosis vaccine followed by delayed anticoccidial intervention in the feed or drinking water) permits the development of immunity and improves performance compared to ionophore treatment alone (5).

At present, there are seven documented *Eimeria* spp. that infect domestic turkeys (*Meleagridis gallopovo* [var. domesticus]) (6,7). Only four of the seven species (*E. meleagrimitis, E. adenoides, E. gallopavonis,* and *E. dispersa*) are more notably pathogenic in commercial turkeys (8). However, infection with multiple species makes it difficult to truly estimate the effects of a single species in the field. For vaccination, turkey integrators have been limited to commercially available live *Eimeria* spp. vaccines that currently do not contain all relevant species. There is evidence of wild turkey fecal samples harboring *Eimeria* spp. that are frequently detected in commercial turkey populations (1). Thus, we sought to investigate the effectiveness of drug-sensitive wild turkey *Eimeria* spp. and amprolium as a candidate bioshuttle program for the commercial turkey industry. Initially, we evaluated an *E. meleagrimitis* vaccine candidate with and without amprolium intervention.

MATERIALS AND METHODS

Experimental design. At day-of-hatch (DOH), turkey poults were allocated into the following treatment groups: 1) non-vaccinated, non-challenged control (NC), 2) non-vaccinated, challenged control (PC), 3) *E. meleagrimitis* candidate vaccine + Amprol (VX + Amprol), or 4) VX. The VX + Amprol treatment group received amprolium (0.024%) in the drinking water from day 10-14. There were two vaccination levels for the vaccinated groups: directly vaccinated (directs) or indirectly vaccinated (contacts). For the vaccinated groups, 50% of the poults assigned to those groups orally received 50 sporulated *E. meleagrimitis* (VX) oocysts immediately prior to placement. The NC, PC, and contacts did not

receive any treatment prior to placement. Each treatment group was housed in a single 7x7ft floor pen with fresh pine shavings (n=60-70 poults/pen). From DOH-day 10, poults for each treatment group were housed to simulate commercial brooding density (0.475 sq. ft./poult). From day 10-termination, density was 0.817 sq. ft./poult. Individual body weights were recorded at placement, day 8, day 23, and day 29 (termination) to determine average body weight gain (BWG). Oocyst shedding during the duration of the study was monitored by collecting fecal and litter samples from day 5-28 post-vaccination. Individual fecal samples were collected from a subset of the directs and contacts in the vaccinated groups. However, pooled fecal samples were collected for NC and PC. Pooled litter samples were collected for each treatment group. To determine fecal and litter OPG, a McMaster chamber was used to enumerate oocysts in each sample (9). At day 23, all poults in the PC and vaccinated groups were orally challenged with 95,000 sporulated *E. meleagrimitis* oocysts. At day 29, or six days post-homologous challenge, macroscopic intestinal lesion scores (duodenum to lower intestine) were evaluated and recorded for each group and vaccination level (n=18-20/treatment/level).

Vaccine candidate. The strain of *E. meleagrimitis* utilized was isolated from a fecal sample collected from a wild turkey in Maine, USA. The sample was submitted to the UADA Poultry Health Laboratory in 2019. Sensitivity to monensin, zoalene, and amprolium was confirmed. A single oocyst derived stock was generated, identity confirmed (PCR and sequencing), and used for vaccination and challenge.

RESULTS AND DISCUSSION

There were no significant effects on DOH-day 8 BWG across all treatment groups (data not shown). DOH-day 23 BWG for the PC group (prior to challenge) was significantly ($P \leq 0.05$) higher than BWG for contacts in the VX + Amprol group. Post-challenge BWG was markedly ($P \leq 0.05$) improved for the direct and contact poults of the VX + Amprol and VX groups compared to the PC. Since this was a pilot study, additional experiments with more replicate pens will be conducted to validate these results. Intestinal lesion scores were evaluated six days post-challenge. Lesion scores were significantly ($P \leq 0.05$) reduced for the directly vaccinated and indirectly vaccinated (contact) turkeys as compared to the NC and PC groups (Figure 1). There were no significant differences in average lesion scores between vaccinated groups or vaccination level. The presence of macroscopic lesion scores in partially immune birds may not fully indicate vaccine-induced protection or lack thereof, as discussed by Chapman et al. (10). Recently, bioshuttle programs have been used in commercial poultry operations. In the current study, drinking water administration of amprolium from day 10-14 reduced average fecal OPG for directs and contact poults (Figure 2). Naive contact poults that did not receive any drug intervention to attenuate oocyst cycling had a sharp increase in fecal OPG from day 12-15 compared to all other groups. The importance of proper coccidiosis vaccination methods was represented by the difference in fecal OPG between contacts and direct of the VX group and between contacts of VX group and contacts of the VX + Amprol group. Large-scale studies will be conducted to evaluate combinations of drug-sensitive wild turkey *Eimeria* spp. as a candidate live coccidiosis vaccine, as a standalone, or implemented with a bioshuttle program. If successful, this will provide the turkey industry with a strategy to control coccidiosis that could be customized based on complex needs.

(The full-length article will be submitted to a relevant journal.**)**

ACKNOWLEDGEMENTS

This study was funded by USDA Animal Health (FY21).

REFERENCES

1. Ruff MD, Schorr RL, Davidson WR, Nettles VF. Prevalence and identity of coccidian in pen-raised wild turkeys. Journal of Wildlife Diseases. 24(4):711-714; 1988.

2. Chapman HD. Milestones in avian coccidiosis research: a review. Poultry Science, 93(3):501–511;2014.

3. Chapman HD. Biochemical, genetic and applied aspects of drug resistance in *Eimeria* parasites of the fowl. Avian Pathology, 26(2):221–244; 1997.

4. Chapman HD, Jeffers TK. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. Int J Parasitol Drugs Drug Resist, 4(3):214-217; 2014.

5. Montoya A, M Quiroz. Field experience in the U.S.A. of a concurrent use of coccidiosis vaccine in combination with ionophores in the feed of broiler chickens. Field Experience: 3628; 2013. URL: http://www.novusint.com/Portals/0/Documents/3628

%20Field%20Experience.pdf. Novus International Inc., St. Louis, MO.

6. McDougald LR, Jeffers TK. *Eimeria tenella* (Sporozoa; Coccidia) gametogony following a single asexual generation. Science, 192:258-259; 1976.

7. Chapman HD. Coccidiosis in the turkey. Avian Pathology, 37(3):205-223; 2008.

8. Lund EE, Farr MM. Coccidiosis of the turkey. Diseases of Poultry, 5th ed. (pp. 1088-1093). Ames, IA: Iowa State University Press; 1965.

9. Conway DP, McKenzie ME. Poultry Coccidiosis: Diagnostic and Testing Procedure. Wiley-Blackwell, Hoboken; 2007.

10. Chapman HD, Roberts B, Shirley MW, Williams RB. Guidelines for evaluating the efficacy and safety of live anticoccidial vaccines, and obtaining approval for their use in chickens and turkeys, Avian Pathology, 34:4, 279-290; 2005.

Figure 1. Cumulative lesion scores six days post-challenge. At day 23, all poults, except for the NC, were orally challenged with *E. meleagrimitis* (95,000 sporulated oocysts/mL). Six days post-challenge (day 29), a subset of the poults from each group and vaccination level (n=18-20/group) were lesion scored. A lesion score of "0" represents a healthy organ whereas a score of "4" represents severe coccidiosis. No lesion scores of 4 were observed. Numbers within columns indicate the number of poults evaluated for each lesion score (0-3). Mean lesion score ± standard error presented above columns. Means further separated using Proc Mixed Analysis (SAS 9.4). [a-c] Different superscripts between treatment groups indicate means differ significantly ($P \leq 0.05$).

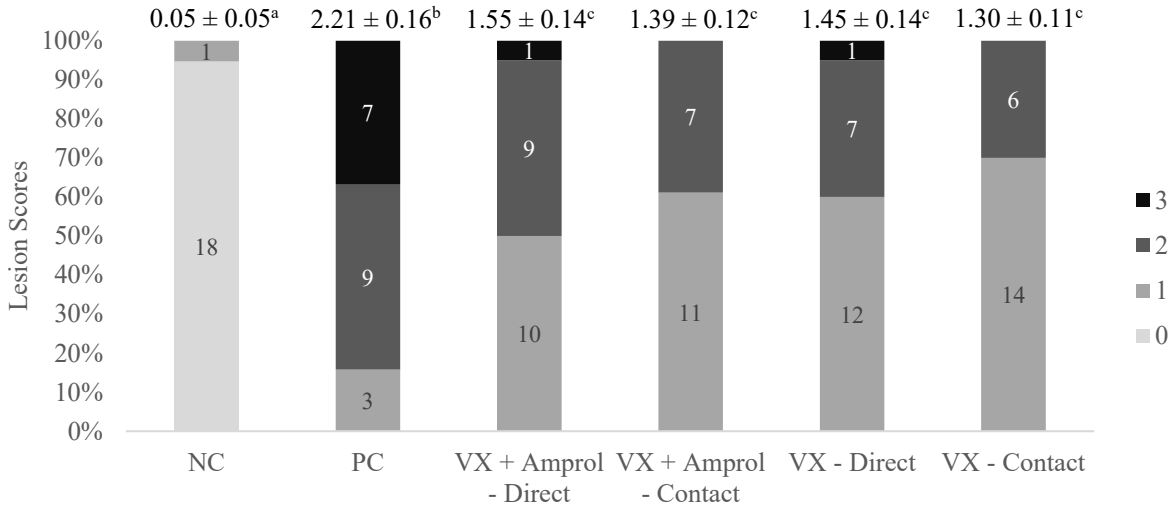
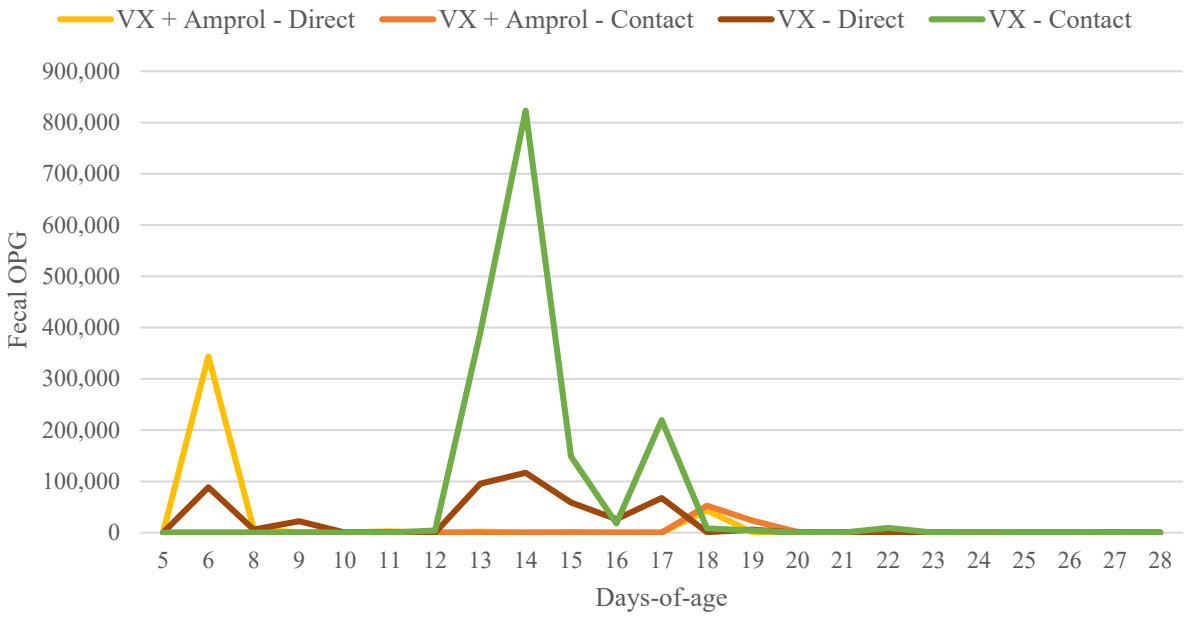


Figure 2. Mean fecal OPG for vaccinated groups by vaccination level (n=3-10 individual fecal samples/group/vaccination level/day). Directly vaccinated poults (directs) orally received 50 *E. meleagrimitis* oocysts at DOH. Contacts were comingled with directs for the duration of the study. VX + Amprol group received amprolium in the drinking water from day 10-14.



EPIDEMIOLOGY OF SPOTTY LIVER DISEASE IN LAYERS: STARTING FROM SCRATCH

P. Groves[A], M. Singh[A], C. Clark[A], and Y. Gao[A]

[A]Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camden, NSW, Australia

SUMMARY

Spotty liver disease (SLD), caused by *Campylobacter hepaticus*, has been a continuing problem in the free-range layer industry for many years. The epidemiology of this disease has not been addressed in a structured sense. A nation-wide analytical epidemiological survey (Case: Control) of the occurrence of SLD in cage-free (free range and barn) layer hens was conducted across Australia in 2020-2021. A major outcome was the identification of the presence of a scratch area in the house as a major risk factor for the occurrence of SLD.

INTRODUCTION

Spotty liver disease (SLD) has been a consistent problem for layer farmers following the increase in popularity of barn and free-range layer farming in many countries. SLD is now known to be caused by *Campylobacter hepaticus* (1,2). SLD may cause mortality of 10% and egg production drops of 25% in cage-free production systems (2). The syndrome is not experienced in cage production. The disease is widespread but not all barn and free-range farms are affected. The epidemiology of SLD has not been studied in depth and the present study was conducted to improve understanding of its occurrence and to search for environmental and management factors which may modify its prevalence.

MATERIALS AND METHODS

The objective of the study was to conduct an analytical epidemiological case control study to identify key determinants for the occurrence of SLD in barn and free-range facilities. Thirty-two rearing farms and twenty-four laying facilities across five states of Australia were surveyed by questionnaire. Twelve cloacal swabs and pooled fecal samples were collected at each visit and examined for detection of *C. hepaticus* by PCR. Individual houses were classified as Cases of SLD or non-cases (Controls) on the basis of the occurrence of an egg production drop and mortality showing clinical and post-mortem signs consistent with SLD and positive detection of *C. hepaticus* from cloacal swabs.

The questionnaire was extensive and covered farming practices, house design, ventilation system, water source, free range management information, nutrition and many other factors.

RESULTS

From the survey there were 18 flocks identified as SLD Cases and six declared as Controls. The mean number of birds with *C. hepaticus* detected from 12 cloacal swabs for the Case flocks was 8.8 (range 4 to 12 positive) while *C. hepaticus* was detected in two of the Control flocks (2 birds positive in each).

Initial univariate analyses identified a zero cell in the questionnaire factor for the presence of flooring type in layer houses (Table 1). In this instance, all houses in the survey that contained a scratch area (i.e. some floor area not covered by slats) were Cases. Houses which had their floors fully covered by slats had only five Case occurrences (45%) and all six Controls.

The scratch area factor confounded other analytical attempts to identify further putative determinant factors and flocks with a scratch area were excluded from further analysis. The data for remaining 11 flocks with fully slatted floors were examined in an attempt to identify factors involved in SLD occurrence in that type of house but the sample size limited statistical precision. Other factors identified as being of potential further interest as increasing risk of SLD in fully slatted houses included smaller flock size (<16000 birds), breed, natural ventilation system, slat type and light type; but these factors showed complete autocorrelation with each other and their contributions to risk of SLD were not separable.

DISCUSSION AND CONSLUSIONS

C. hepaticus can be detected in flocks that are not exhibiting clinical SLD but at a lower prevalence than Case flocks.

The presence of a scratch area in the house appears as a major risk factor for the occurrence of SLD in an adult cage-free layer flock. SLD is regarded as having a fecal-oral transmission method (1,3) and the complete coverage of the house floor with slats would provide some protection from bird contact with fresh feces. The complete association with having a scratch area confounded further analyses in those houses and the remaining sample size did not allow individual identification of other factors which may modify the risk of SLD where the floor is fully covered by slats. This will require a larger scale focussed survey in fully slatted houses to define possible factors, which is currently underway.

(The full-length article will be published in a peer reviewed journal in the near future.)

ACKNOWLEDGEMENTS

The project was funded by Australian Eggs Limited.

REFERENCES

1. Moore RJ, Scott PC, Van TTH. Spotlight on avian pathology: Campylobacter hepaticus, the cause of Spotty Liver Disease of layers. *Avian Pathology* 48”285-287. 2019.
2. Courtice JM, Mahdi LK, Groves PJ, Kotiw M. Spotty Liver Disease: A review of an ongoing challenge in commercial free-range egg production. *Veterinary Microbiology* 227: 112-118.
3. Van, TTH, Gor M-C, Anwar A, Scott PC, Moore RJ. Campylobacter hepaticus, the cause of spotty liver disease in chickens, is present throughout the small intestine and caeca of infected birds. *Veterinary Microbiology*. 207, 226-230. 2017.

Table 1. Contingency table for presence of a scratch area in the layer house and association with occurrence of SLD.

Exposure level	No. SLD Case flocks	No. Control flocks	Odds Ratio	Fisher’s exact* P=
Scratch area in house	13	0	Infinite	0.009
Full slat cover	5	6		

*An expected value was <5, hence Fisher’s exact, 2-tail test used to determine significance

A UNIVERSAL *ESCHERICHIA COLI* SUB-UNIT VACCINE PROTECTS BROILERS FROM E. COLI 078 CHALLENGE

J. Hall[A] E. Gumina[B], and S. Layton[A,B]

[A]Vetanco USA, Department of Research and Development, Saint Paul, MN, 55114 USA
[B]Vetanco S.A., Department of Biotechnology, Villa Martelli, Providence of Buenos Aires, B1603CMA, Argentina

SUMMARY

A universal oral *Escherichia coli* sub-unit vaccine was developed and tested in broilers. Two preliminary efficacy trials (10-15 birds/group) have been conducted using intraperitoneal (IP), intratracheal (IT), and oral methods of challenge. The vaccine was orally gavaged at d three and 17 of life and all birds were challenged seven days post-booster dose with *E. coli* 078 at 7 and 8 log CFU/bird and necropsied four to seven days post-challenge. In both trials, the vaccine provided protection from mortality and limited pathological damage to internal organs. Importantly, in the oral-challenge group, the vaccine significantly reduced the number of ceca that were positive for *E. coli*, and within those ceca containing *E. coli*, it significantly reduced the quantity of *E. coli* present. This vaccine may serve as a useful tool to reduce *E. coli* infections in poultry and prevent animal mortality during production and food-borne illnesses in humans.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) is a heterogenous group of pathogenic *E. coli* serotypes commonly associated with avian colibacillosis, a multi-systemic disease with clinical presentation as airsacculitis, cellulitis and synovitis, salpingitis, peritonitis, septicemia, or other disease of tissues, which often result in death (1-5). It is a major disease of poultry worldwide and results in significant loss of production and high levels of mortality within flocks (2, 6, 7). Further, certain APEC serotypes may pose a food-borne illness risk to humans (2). *E. coli* is a commensal of the poultry intestinal microbiome, but acquisition and vertical transmission of specific virulence factors, often on mobile genetic elements, promote the pathogenicity of *E. coli* (4, 8-14). APEC are a sub-group of extraintestinal pathogenic E. coli (ExPEC), demarcating the group from Intestinal Pathogenic *Escherichia coli*, IPEC (15), It is believed that APEC strains originate in the intestines of the bird, with detection of APEC as early as one-day of age (16). Ultimately, on their own, or induced by stressors, the APEC translocate from the intestines into the intraperitoneal cavity and disseminate to various tissues causing disease and death (5, 15, 16).

Administration of antibiotics and vaccination are common therapeutic and prophylactic treatments for colibacillosis, respectively. Unfortunately, resistance to almost all classes of antibiotics is now common for *E. coli* as a species, not just APEC (2, 10, 17-21). Worldwide, there are several commercial poultry-specific *E. coli* vaccines available, but most rely on specific serotypes, thus they only contain a relatively small fraction of the possible antigens found within the heterogeneic APEC group and fail to fully protect the birds, providing at best, serotype specific protection. The lack of efficacious cross-protection among vaccines means novel vaccination strategies must be explored and developed, especially as broiler production, particular in the USA, moves to a "No Antibiotic Ever" format (22). Many of these experimental vaccines rely on identification of common virulence or primary immunogenic factors (9, 23) or complicated mixtures of isolates and rely on, likely, commercially infeasible purification processes (24). Others, while highly protective, have expensive production processes that are not financially suitable for broiler production (25). A highly cross-protective, inexpensively produced, and easily administered colibacillosis vaccine is greatly needed to meet the demands of the changing broiler market, further reduce use of antibiotics in production, and maximize profits for growers.

The data herein describes the evaluation of a universal experimental *E. coli* subunit vaccine (ESV). The antigen and porcine optimized formulation has previously been tested and shown to protect neonatal and weanling piglets from a dual a enterotoxigenic *E. coli* (ETEC) challenge (26). These initial experimental trials of a poultry optimized ESV formulation utilized our previous experience with coccidiosis (27) . The vaccine platform is based on a proprietary GRAS-listed *Bacillus subtilis* antigen production system,

which was engineered to produce a highly conserved *E. coli* antigen integral to cellular survival. After fermentation, the antigen is encapsulated in a natural polysaccharide, producing antigen filled microcarriers, which can be diluted into the drinking water system or directly administered orally or nasally (26, 27) delivering direct application to the mucosa. The production of antigen-specific secretory IgA in the mucosa, the primary portal of entry and reservoir of pathogenic *E. coli*, limits infection (16, 26). From there, vaccine induced immunity is generated throughout the mucosal immune system (28). Ultimately, the immune response becomes systemic, with the production antigen-specific serum IgY(G)s and T-cells (29), which may limit the translocation of pathogenic *E. coli* from the gastrointestinal tract (GIT) into the abdomen and surrounding tissues. This ESV is easily administered, cost-efficient, and safe and effective and offers the potential protection against the many *E. coli* serotype. The further development of this vaccine is greatly needed to combat this pervasive disease, promote responsible antimicrobial stewardship and animal husbandry practices.

METHODS

General methodologies

Housing conditions and husbandry. This experiment was conducted at the experimental farm of Vetanco USA. The broiler barn is an approximately 1200 square feet enclosed barn with a concrete floor. An enclosed 144 square feet floor pen was equipped with two feeders and two waters. Heat was provided via heating lamps hung from the rafters and fans are placed at entry ways and over the birds for heat relief. The stocking density was below the National Chicken Council Animal Care Guidelines(https://www.nationalchickencouncil.org/wp-content/uploads/2021/02/NCC-Animal-Welfare-Guidelines_Broilers_Sept2020.pdf) and all procedures were consistent with 9 CFR 108.10 and 117 of the United States Department of Agriculture regulations.

Animal source and diets. One hundred one-day-old mixed sex Cornish cross broilers (Hoover Hatcheries, Rudd, IA) were placed in the floor pen and allowed to acclimate for 48 hours prior to primary vaccination. Purina Start and Grow Medicated (amprolium, a coccidiostat) Chick Feed Crumbles, as well as water were provided ad libitum. Husbandry conditions such as environmental temperature and the light program were adjusted to the recommended guidelines of the genetic line.

Challenge strain. The WT E. coli 078 strain (chi7122, PMID 12506201) was kindly provided by Mid-Central Research and Outreach Center (Willmar, MN, Dr. T. Johnson) (30). The isolate was cultured overnight and frozen in glycerol stocks at -80 °C for storage. The day before the challenge, a sterile pipette was used to scrape the frozen stock and inoculate tryptic soy broth (TSB), which was cultured overnight at 37 °C at 225 RPM. The following morning the culture was diluted 1:100 into fresh TSB and cultured until mid-log phase. The cultured was centrifuged at 4 °C and was washed and re-centrifuged thrice in ice-cold PBS. The cell pellet was resuspended in ice-cold PBS using a volume ½ of the original culture volume and diluted to the indicated concentration based on a previously established CFU/mL:$OD600_{nm}$ curve analysis for the isolate. The final concentration of each challenge dilution was determined retrospectively on tryptic soy agar with 5% sheep's blood.

Vaccination. At three days of age, the chicks were divided in half with one half randomly assigned to vaccination group and the other half assigned the untreated control group. Within the vaccination group, each chick was orally dosed with 0.2 mL of the ESV by oral gavage. Subsequently, each group was further randomly divided into three groups of at least 15 chicks each and birds were double leg banded with one of six colored bands (trial 1) or neck tagged with one six colored and numbered tags (trial 2). Once all birds were tagged, they were comingled in the floor pen. On d 17, the groups were sorted, and the three groups designated for vaccination were given a second 0.2 mL dose of ESV by oral gavage. Birds that died prior to challenge were not replaced.

Necropsy and pathology scoring. Necropsy was performed at time of death discovery or four or six days post-challenge as indicated. Any bird incapable of accessing food or water under their own will was humanely euthanized by cervical dislocation and counted as mortalities in the statistical analysis. At necropsy, lesions were assessed for the liver, heart, and air sac. The scoring matrix for air sacs are based on descriptions according to Soleymani et al. (31) with slight modifications as follows: 1: no lesions, 2: cloudiness of air sacs and/or thickened air sac membranes, 3: "meaty" appearance of membranes with large accumulations of a cheesy exudate in one or both air sacs. The pericardial and perihepatic lesions were scored with slight modifications. For perihepatic lesions, 1 indicates no visible lesions; 2 indicates definite fibrination on the surface of the liver; and 3 indicates extensive fibrination, adhesions, liver swelling, and necrosis and for pericardial lesions, scoring was as follows: 1: no visible lesions, 2: excessive clear or cloudy fluid in the pericardium, and 3: extensive fibrination in the pericardial cavity.

Statistical analysis. All data entry and analysis were conducted in GraphPad Prism v 9.3.0. Differences of means was considered significant at α

≤ 0.5. Absolute mortality, the presence/absence of pathology, and positive/negative status of ceca were analyzed using a Fisher's Exact test. The organ scores were compared between controls and vaccinated birds at the same challenge concentration using the non-parametric Mann-Whitney Test in Experiment 1. The organ scores between controls and vaccinated birds within the same challenge route were analyzed by two-way ANOVA with multiple comparisons using a post-hoc Šidák correction.

Experiment 1

Challenge. The model and challenge route utilized in Experiment 1 was based on a previously described intraperitoneal challenge model for E. coli O78 (25). Briefly, seven days post booster dose administration (d 24 of life), all birds were intraperitoneally challenged with the respective challenge dose using a 1-cc syringe with a 1-inch 23-guage needle to inject 1 mL of the APEC preparation into the peritoneal cavity between the tip of keel bone and cloaca (25). Each control and vaccination treatment group were matched with the following APEC concentration: ~6 log, ~7 log, and ~8 log CFU/mL. Six days post-challenge, as birds remaining alive were humanely euthanized and necropsied for organ scoring as described.

Blood was collected from the wing vein from five birds/group in serum separator tubes and stored on ice until centrifugation and serum harvesting in the laboratory.

Experiment 2

Selection for a nalidixic acid resistant APEC O78 isolate. To facilitate re-isolation and quantification of the APEC O78 isolate from organs, a nalidixic acid resistant (Nal^R) strain was isolated. To selection for the Nal^R strain, an overnight culture grown in LB broth of WT O78 isolate was diluted 1000-fold into fresh LB containing 2-fold dilutions of nalidixic acid starting at 250 ug/mL were plated in triplicate in a 96-microwell format. The plate was covered with a Breathe Easy® Sealing Membrane and was incubated for 18 hours at 37 °C in a TECAN INFINITE M200 PRO. Every 15 minutes the plate was shaken orbitally and the optical density at $\lambda = 600$ nm was measured. The following morning, the kinetic $OD600_{nm}$ measurement were plotted and compared to the growth of the bacteria without antibiotics from the same plate. Concentrations of nalidixic acid at and below 62.5 ug/mL minimally inhibited growth of the WT strain. At 125 and 250 ug/mL, the lag phase of the strain as greatly increased, but ultimately the isolate grew to a similar density as the WT without nalidixic acid present. The cultures from the 250 ug/mL microwells were separately streaked onto LB agar containing 250 ug/mL of nalidixic acid. The following morning, several plates had well isolated colonies. Several of these colonies were subsequently cultured in LB broth with up to 1000 ug/mL of nalidixic acid, indicating the creation of a E. coli O78 isolate with a high level of resistance to nalidixic acid.

Enhancement of APEC O78 Nal^R pathogenicity. As the newly isolated APEC O78 Nal^R had been passaged several times in the laboratory, the isolate was passaged through broiler chicks to try to increase the isolates pathogenicity. Overnight cultures were diluted to ~9 log in PBS for IP injection (1mL) and intratracheally gavages (0.5 mL). The isolate was passaged through three chicks per method of challenge. Twenty-four to 48 hours after each treatment, chicks were humanely euthanized, and their air sacs were sterilely swabbed and a section of the liver and both lungs were sampled. The air sac swabs were streaked onto EMB agar plates containing 500 ug/mL of a nalidixic acid (EMB-Nal). Liver and lung were mixed 1:2 (w/v) with sterile PBS and serial dilutions were plated on EMB-Nal. All plates were incubated overnight at 37 °C. The isolate, chi7122-Nal^R, used in Experiment 2 was isolated from the liver of an IP injected chick, which had a liver score 3 and an air sac score 2. None of the chicks died prior to euthanasia.

Challenge. The APEC O78 Nal^R was cultured and prepared as previously described, but 250 ug/mL of Nal was added to the TSB during cultivation.

Since the ESV is an orally delivered vaccine and mucosal immunity is an important mechanism of the vaccine's potential protection, two additional challenge methods, intratracheal (IT) and oral/gastrointestinal (Oral) were included in this trial to mimic a mucosal route of infection, in addition to the IP route. For the IP challenge, APEC Nal^R at 7 log CFU/mL was injected intraperitoneally as described previously seven days post-booster dose. For the oral challenge, a single 0.5 mL volume of APEC O78 Nal^R at ~8 log CFU/mL was orally gavaged seven days post-booster dose. For the IT challenge, starting at 5 days post-booster dose, birds in this challenge group were given 0.5 mL of APEC O78 Nal^R at 8 log CFU/mL for three successive days using a curved 2-inch stainless steel canula directly in the trachea of the bird. Birds that died or were humanely euthanized to alleviate pain and suffering were immediately necropsied and organ scored. Four days after the final challenge, birds remaining alive in all groups were humanely euthanized and necropsied. Organ scoring was done as described previously. The spleen and a section of the liver from all bird was aseptically harvested for presence/absence detection of O78 Nal^R on EMB-Nal. Further, in the IT groups, both lungs were aseptically harvested for presence/absence detection of O78 Nal^R on EMB-Nal and the right cecum, relative to the cecal tonsil junction, was

harvested for quantification of O78 Nal^R on MacConkey Agar-Nal in the orally challenged groups. The spleen and liver of individual birds was combined and diluted 1:5 (w/v) with sterile PBS and 100 µl of each sample was allowed to drip dry down angled EMB-Nal plates in square petri dishes. The lungs and ceca were processed in a similar manner, but the ceca were serially diluted to quantify the Nal^R *E. coli*. Only birds that were necropsied at the end of the trial were included in the *E. coli* presence/absence analysis for organs.

RESULTS

The primary purpose of the Experiment 1 was to reproduce the IP challenge model in our facility as described by Cox et al. (25). Secondarily, we compared the mortality of broilers and organ pathology between the control and ESV-vaccinated groups. At 6 Log_{10} CFU/mL a similar number of the control and ESV birds died and organ pathology consistent with colibacillosis (Table 1), while the mean organ scores were numerically lower for the ESV birds compared to the control birds (Figure 1A). At 7 Log_{10} CFU/mL, the control birds had a 31% mortality rate, while none of the vaccinated birds died (Table 1). Further, the were significantly fewer birds in the ESV group with colibacillosis organ pathology compared to the control group (Table 1). The mean organ pathology scores were lower in the ESV group, with near ablation of air sac pathology in the ESV group compared to control group ($p = 0.015$) (Figure 1A). At 8 Log_{10} CFU/mL, a 38% mortality rate was observed on the control birds, which is lower than was what described (Table 1) (25) , with colibacillosis pathology observed in 92% and 75% of the control and ESV birds, respectively. Again, the mean organ scores were numerically lower in the ESV birds compared to the control birds (Figure 1A).

In Experiment 2, the purpose was to reproduce the IP challenge results at 7 Log_{10} and evaluate an intratracheal and oral gavage challenge method as to model a direct challenge to the mucosa. Cox et al reported a single 1 mL IT challenge at 9 Log_{10} CFU with their nalidixic acid resistant chi7122 derivative did not cause mortality or pathology (25), though it has been reported as replicable route of challenge for other APEC isolates (32), it may not be the most suitable route for this strain thus, we investigated the use of three consecutive days of an IT challenge and a single oral gavage challenge as described in the Methods.

In the orally challenged group, an equal number of birds died (Table 2) and there was not a statistical difference in the presence of colibacillosis pathology between the control or ESV birds (Table 3). At the end of the trial, the right ceca of the 13 remaining birds were diluted and plated on MacConkey Agar-Nal to differentiate the *E. coli* from contaminating *Salmonella* spp. and quantify nalidixic acid resistant *E. coli.* The ESV vaccinated birds had significantly fewer ceca positive for nalidixic acid resistant *E. coli* compared to the control group (Table 3) and the mean CFU counts for the ESV vaccinated group's ceca was significantly lower than the control group's ceca (Figure 1B).

In the IP challenged groups, the mortality was reduced compared to the results of Experiment 1, with one bird dying in the control group and none in the ESV group. Overall, there were significantly fewer ESV-vaccinated birds with colibacillosis pathology in the heart, liver, or air sac compared to the control group. Specifically, the mean pathology score of the air sac and heart was significantly reduced in the ESV-vaccinated birds relative to the control group birds (Figure 1C), but there was not a difference in the detection of nalidixic acid resistant *E. coli* in the liver or spleen between the groups (Table 3).

None of the birds died in the 3xIT challenged groups (Table 3), but significantly more birds in the control group had colibacillosis pathology of the heart, liver, or air sac. Specifically, the mean score of the heart was significantly less in the ESV-vaccinated group compared to the control group whereas the mean air sac score was also numerically lower (Figure 1D). Further, only one bird from each group had detectable nalidixic acid resistant *E. coli* in the lungs at necropsy (Table 3).

DISCUSSION

An inexpensively produced and easily applied vaccine with broad serotype efficacy is greatly need for the poultry industry as consumer demand, government regulations (2015 FDA VFD), and the advancement of antibacterial resistance further reduces the availability of therapeutics to treat colibacillosis (21, 22). The sub-unit antigen vaccine evaluated here is produced by a Generally Recognized as Safe (GRAS) organism, *Bacillus subtilis*, which is easily fermented with minimal inputs and downstream processing. The vaccine is essentially two components; the fermented antigen and the encapsulation media, which are mixed and produce the final formulated vaccine. The antigen is highly conserved across pathogenic *E. coli*; by employing an orally delivered vaccine, secretory IgA and the mucosal immune system can be activated to target this essential protein (26), in so doing it limits infection and replication of *E. coli* within the intestinal tract of the birds (16).

The studies described here evaluated three routes of challenge infection and the efficacy of an

experimental subunit *E. coli* vaccine (ESV). With regards to each challenge route, each posse their own benefits and drawbacks. The IP route appears to have the highest level of mortality (25), but it appears to be dependent on the virulence of the serotype and laboratory passaging of the isolate. Intraperitoneal injections give the *E. coli* direct access to a normally sterile environment and bypass much of the immune system, specifically the mucosal immune system important for protection induced by this ESV. Nonetheless, the ESV was able to reduce mortality and colibacillosis pathology after an IP challenge of 7 Log_{10} CFU/mL of chi7122. An intratracheal route of challenge directly exposes the mucosa of the broilers to the *E.* coli and may allow the bacteria to translocate from the lungs to the bloodstream and air sac. Indeed, the ESV reduced colibacillosis pathology in broilers and protected the heart and air sac in many of the vaccinated birds. It is believed that the GIT is the primary reservoir of *E. coli* in poultry (2, 16, 32); thus, a direct oral challenge would replicate contamination of the broiler from environmental sources (e.g. feces) (16), An efficacious mucosal vaccine, which prevents colonization of the broiler, would likely lead to a reduction in colibacillosis. Significantly, vaccination with the ESV reduced cecal colonization by nalidixic-resistant *E. coli* after a direct oral challenge. Further, 50% fewer birds were observed with colibacillus pathology. Regardless of the route of challenge, the ESV evaluated displays efficacy against an APEC 078 serotype and its composition provides it the potential to have broad serotype efficacy, which needs further evaluation. As *E. coli* are an important zoonotic and food-borne pathogen as well as major economic burden on the poultry industry, an inexpensive and efficacious *E. coli* vaccine is desperately needed. The ESV evaluated here has been shown to protect broilers from a variety of challenge routes and its continued development and ultimate availability will hopefully provide broiler producer an inexpensive and easily administered *E. coli* vaccine.

REFERENCES

1. Nolan LK, Barnes HJ, Vaillancourt J-P, Abdul-Aziz T, Logue CM. Colibacillosis. *Diseases of Poultry*. Ames (IA): Wiley-Blackwell. p. 751-805; 2013.

2. Kathayat D, Lokesh D, Ranjit S, Rajashekara G. Avian Pathogenic Escherichia coli (APEC): An Overview of Virulence and Pathogenesis Factors, Zoonotic Potential, and Control Strategies. *Pathogens.* 10:467; 2021 Apr 12.

3. Guabiraba R, Schouler C. Avian colibacillosis: still many black holes. *FEMS Microbiol Lett.* 362:fnv118; 2015 Aug.

4. Pires-Dos-Santos T, Bisgaard M, Christensen H. Genetic diversity and virulence profiles of Escherichia coli causing salpingitis and peritonitis in broiler breeders. *Vet Microbiol.* 162:873-880; 2013 Mar 23.

5. Collingwood C, Kemmett K, Williams N, Wigley P. Is the Concept of Avian Pathogenic Escherichia coli as a Single Pathotype Fundamentally Flawed? *Front Vet Sci.* 1:5; English. 2014 2014.

6. Wibisono FJ, Sumiarto B, Kusumastuti TA. Economic Losses Estimation of Pathogenic Escherichia coli Infection in Indonesian Poultry Farming. *Buletin Peternakan.* 42: 2018.

7. Landman WJ, van Eck JH. The incidence and economic impact of the Escherichia coli peritonitis syndrome in Dutch poultry farming. *Avian Pathol.* 44:370-8; 2015 Oct.

8. Dziva F, Stevens MP. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts. *Avian Pathol.* 37:355-66; 2008 Aug.

9. Olsen RH, Christensen H, Bisgaard M. Comparative genomics of multiple plasmids from APEC associated with clonal outbreaks demonstrates major similarities and identifies several potential vaccine-targets. *Vet Microbiol.* 158:384-93; 2012 Aug 17.

10. Kariyawasam S, Johnson TJ, Debroy C, Nolan LK. Occurrence of pathogenicity island I(APEC-O1) genes among Escherichia coli implicated in avian colibacillosis. *Avian Dis.* 50:405-10; en. 2006 Sep.

11. Johnson TJ, Nolan LK. Pathogenomics of the virulence plasmids of Escherichia coli. *Microbiol Mol Biol Rev.* 73:750-74; 2009 Dec.

12. Johnson TJ, Jordan D, Kariyawasam S, Stell AL, Bell NP, Wannemuehler YM, Alarcon CF, Li G, Tivendale KA, Logue CM*, et al.* Sequence analysis and characterization of a transferable hybrid plasmid encoding multidrug resistance and enabling zoonotic potential for extraintestinal Escherichia coli. *Infect Immun.* 78:1931-42; 2010 May.

13. Janben T, Schwarz C, Preikschat P, Voss M, Philipp HC, Wieler LH. Virulence-associated genes in avian pathogenic Escherichia coli (APEC) isolated from internal organs of poultry having died from colibacillosis. *Int J Med Microbiol.* 291:371-8; 2001 Nov.

14. Johnson TJ, Siek KE, Johnson SJ, Nolan LK. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian Escherichia coli strains. *J Bacteriol.* 188:745-58; 2006 Jan.

15. Sora VM, Meroni G, Martino PA, Soggiu A, Bonizzi L, Zecconi A. Extraintestinal Pathogenic Escherichia coli: Virulence Factors and Antibiotic

Resistance. *Pathogens (Basel, Switzerland).* 10:1355; eng. 2021.

16. Ewers C, Antão E-M, Diehl I, Philipp H-C, Wieler LH. Intestine and Environment of the Chicken as Reservoirs for Extraintestinal Pathogenic Escherichia coli Strains with Zoonotic Potential. EN. 2009 2009-January-1.

17. Pavez-Munoz E, Gonzalez C, Fernandez-Sanhueza B, Sanchez F, Escobar B, Ramos R, Fuenzalida V, Galarce N, Arriagada G, Neira V*, et al.* Antimicrobial Usage Factors and Resistance Profiles of Shiga Toxin-Producing Escherichia coli in Backyard Production Systems From Central Chile. *Front Vet Sci.* 7:595149; 2020 01/15/2021.

18. Salinas L, Cardenas P, Johnson TJ, Vasco K, Graham J, Trueba G. Diverse Commensal Escherichia coli Clones and Plasmids Disseminate Antimicrobial Resistance Genes in Domestic Animals and Children in a Semirural Community in Ecuador. *mSphere.* 4: EN. 2019 May 22.

19. Muloi D, Kiiru J, Ward MJ, Hassell JM, Bettridge JM, Robinson TP, van Bunnik BAD, Chase-Topping M, Robertson G, Pedersen AB*, et al.* Epidemiology of antimicrobial-resistant Escherichia coli carriage in sympatric humans and livestock in a rapidly urbanizing city. *Int J Antimicrob Agents.* 54:531-537; 2019 Nov.

20. Hayer SS, Rovira A, Olsen K, Johnson TJ, Vannucci F, Rendahl A, Perez A, Alvarez J. Prevalence and trend analysis of antimicrobial resistance in clinical Escherichia coli isolates collected from diseased pigs in the USA between 2006 and 2016. *Transbound Emerg Dis.* 67:1930-1941; 2020 Feb 25.

21. Sargeant JM, Bergevin MD, Churchill K, Dawkins K, Deb B, Dunn J, Logue CM, Novy A, O'Connor AM, Reist M*, et al.* The efficacy of antibiotics to control colibacillosis in broiler poultry: a systematic review. *Anim Health Res Rev.* 20:263-273; 2019 Dec.

22. Fancher CA, Zhang L, Kiess AS, Adhikari PA, Dinh TTN, Sukumaran AT. Avian Pathogenic Escherichia coli and Clostridium perfringens: Challenges in No Antibiotics Ever Broiler Production and Potential Solutions. *Microorganisms.* 8: 2020 Oct 6.

23. Lynne AM, Kariyawasam S, Wannemuehler Y, Johnson TJ, Johnson SJ, Sinha AS, Lynne DK, Moon HW, Jordan DM, Logue CM*, et al.* Recombinant Iss as a potential vaccine for avian colibacillosis. *Avian Dis.* 56:192-9; 2012 Mar.

24. Hu R, Li J, Zhao Y, Lin H, Liang L, Wang M, Liu H, Min Y, Gao Y, Yang M. Exploiting bacterial outer membrane vesicles as a cross-protective vaccine candidate against avian pathogenic Escherichia coli (APEC). *Microb Cell Fact.* 19:119; 2020 Jun 3.

25. Cox GJM, Griffith B, Reed M, Sandstrom JD, Peterson MP, Emery D, Straub DE. A Vaccine to Prevent Egg Layer Peritonitis in Chickens. *Avian Dis.* 65:198-204; 2021 Mar.

26. Jabif MF, Gumina E, Hall JW, Hernandez-Velasco X, Layton S. Evaluation of a Novel Mucosal Administered Subunit Vaccine on Colostrum IgA and Serum IgG in Sows and Control of Enterotoxigenic Escherichia coli in Neonatal and Weanling Piglets: Proof of Concept. *Front Vet Sci.* 8:640228; English. 2021 2021-February-11.

27. Gumina E, Hall JW, Vecchi B, Hernandez-Velasco X, Lumpkins B, Mathis G, Layton S. Evaluation of a subunit vaccine candidate (Biotech Vac Cox) against Eimeria spp. in broiler chickens. *Poult Sci.* 100:101329; 2021 Sep.

28. Ogra PL, Faden H, Welliver RC. Vaccination strategies for mucosal immune responses. *Clin Microbiol Rev.* 14:430-45; eng. 2001 Apr.

29. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med.* 11:S45-53; En. 2005 Apr.

30. Dozois CM, Daigle F, Curtiss R, 3rd. Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic Escherichia coli strain. *Proc Natl Acad Sci U S A.* 100:247-52; 2003 Jan 7.

31. Soleymani S, Tavassoli A, Hashemi Tabar G, Kalidari GA, Dehghani H. Design, development, and evaluation of the efficacy of a nucleic acid-free version of a bacterial ghost candidate vaccine against avian pathogenic E. coli (APEC) O78:K80 serotype. *Vet Res.* 51:144; En. 2020 Dec 9.

32. Antão E-M, Glodde S, Li G, Sharifi R, Homeier T, Laturnus C, Diehl I, Bethe A, Philipp H-C, Preisinger R*, et al.* The chicken as a natural model for extraintestinal infections caused by avian pathogenic Escherichia coli (APEC). *Microbial Pathogenesis.* 45:361-369; 2008 2008/11/01/.

Figure 1. Organ scoring and cecal *E. coli* Nal^R quantification from Experiments 1 and 2. (A) The mean pathology score of the heart, liver, and airsac from control or ESV-vaccinated birds 6 days post intraperitoneal challenge using three different concentrations of WT APEC O78. (B) Quantification of nalidixic acid resistant *E. coli* from the right ceca 6 days post oral challenge with chi7122-NalR from control or ESV-vaccinated birds. Data represents the mean ± SD from all birds within each group and the absolute quantification of positive ceca shown as enclosed dots. Individual organ pathology score of the heart, liver, and airsac from control or ESV-vaccinated birds 6 days post (C) intraperitoneal challenge and 4 days post (D) three consecutive intratracheal challenged.

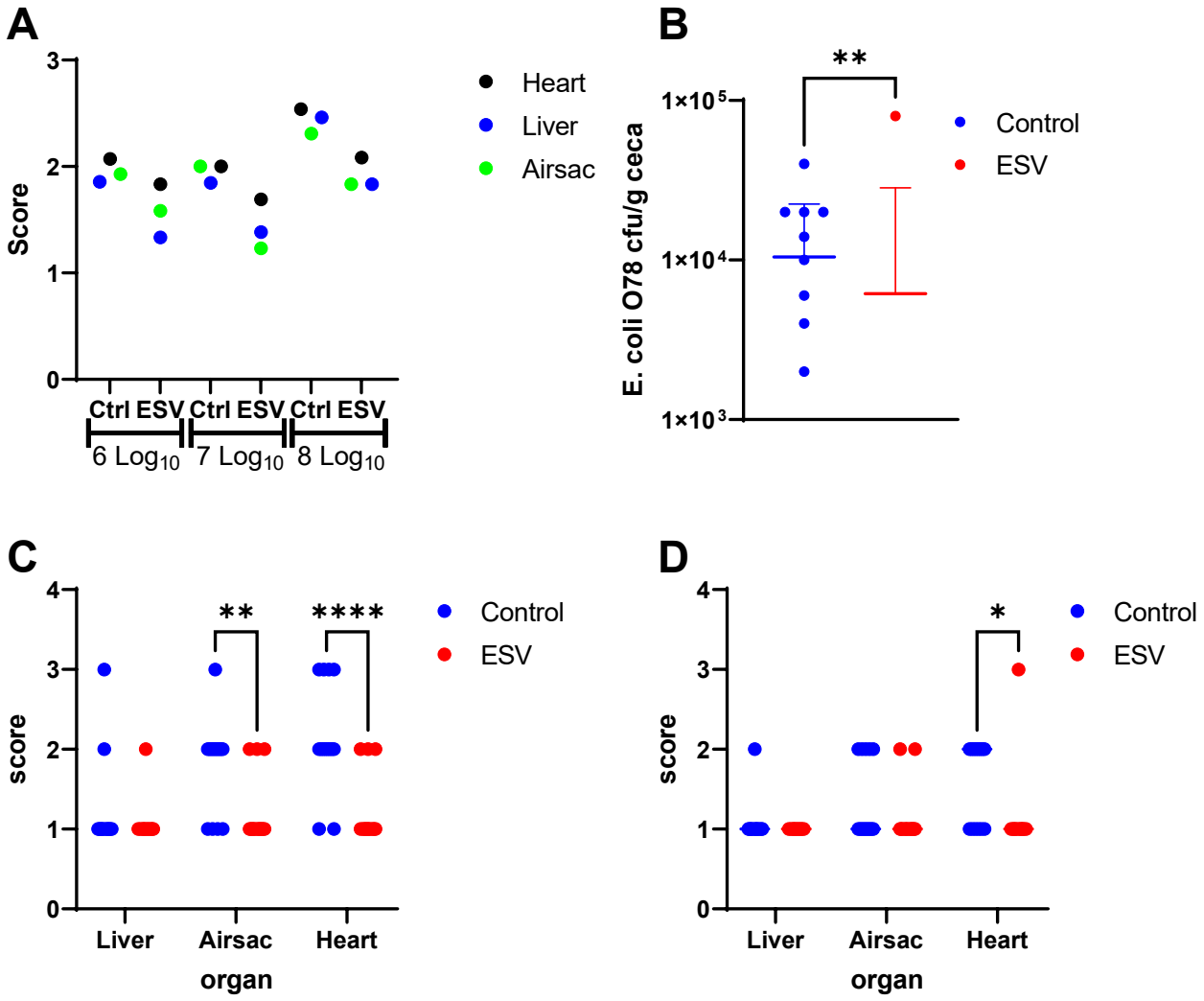


Table 1. Mortality and the presence of organ pathology of intraperitoneal challenged broilers in Experiment 1.

	6 Log_{10}		7 Log_{10}		8 Log_{10}	
	control	ESV	control	ESV	control	ESV
Mortality[A]	2/12 (17%)	2/13 (15%)	4/13 (31%)	0/13 (0%*)	5/13 (38%)	2/12 (17%)
Pathology[B]	10/12 (83%)	9/13 (69%)	12/13 (92%)	6/13 (46%*)	12/13 (92%)	9/12 (75%)

*Fisher's Exact test
[A]A bird found dead post-challenge but prior to end of trial euthanasia.
[B] Sum of pathology scores: no pathology in heart, airsac or liver = 0, Any pathology in heart, airsac or liver = 1.

Table 2. Mortality and the presence of organ pathology of intraperitoneal challenged broilers in Experiment 2.

	IP		3xIT		oral	
	control	ESV	control	ESV	control	ESV
Mortality[A]	1/14 (7%)	0/15 (0%)	0/14 (0%)	0/15 (0%)	2/15 (13%)	2/15 (13%)
Pathology[B]	12/14 (86%)	4/15 (27%*)	9/14 (64%)	2/15 (13%*)	4/15 (27%)	2/15 (13%)

*Fisher's Exact test
[A]A bird found dead post-challenge but prior to end of trial euthanasia.
[B] Sum of pathology scores: no pathology in heart, airsac or liver = 0, Any pathology in heart, airsac or liver = 1.

Table 3. Number and percent of total of specific organs positive for nalidixic resistant *E. coli* harvested at necropsy in Experiment 2.

	IP		3xIT		oral	
organ	control	ESV	control	ESV	control	ESV
liver/spleen	2/14 (14%)	1/15 (7%)	1/15 (7%)	2/15 (13%)	0/13 (0%)	0/13 (0%)
lungs			1/15 (7%)	1/15 (7%)		
right ceca					9/13 (69%)	1/13 (8%*)

PATHOGENICITY OF THE CANADIAN DELMARVA (DMV/1639) STRAIN OF INFECTIOUS BRONCHITIS VIRUS (IBV) FOR LAYING HENS

M. H. Hassan, A. Ali, S. Buharideen, S. Najimudeen, and M. Faizal Abdul-Careem

Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Alberta T2N 4N1, Canada

SUMMARY

Infectious bronchitis virus (IBV) can significantly impact the reproductive performance of laying hens. The infecting IBV strain, age at infection and status of immunity are the major factors that influence the outcome regarding the induction of reproductive disease. Over the past few years, DMV/1639 strain has been identified in flocks presenting with false layer syndrome in Eastern Canada. The aim of this study was to investigate the pathogenicity of the DMV/1639 strain for the reproductive tract of young and adult laying hens. One-day-old and 29-week-old specific pathogen-free (SPF) hens were challenged oculo-nasally with the Canadian DMV/1639 strain. Infection of the young chickens showed wide tissue tropism to the respiratory, urinary, genital, and alimentary tracts at 7 days post-infection (dpi). Necropsy conducted at 16 weeks of age showed marked cystic lesions in the oviduct. Microscopical investigation revealed epithelial sloughing, mononuclear cell infiltrations, and cystic dilated glands in the oviduct. Virus persistence was detected in the cecal tonsils with continuous cloacal viral shedding. In the infected adult hens, the egg production dropped to 40% at 5 dpi. Necropsy conducted at 10 dpi showed regression of the ovary and atrophy of the oviduct. Histopathological changes involved heterophilic and mononuclear cell infiltrations, sloughed epithelium, and degenerated glands in the oviduct. Overall, the study shows that infection of young and adult chickens with the Canadian DMV/1639 strain of IBV induces significant reproductive tract pathologies and negatively impacts layer reproductive performance.

INTRODUCTION

The Canadian DMV/1639 strain of infectious bronchitis virus (IBV) has recently been identified in several layer flocks with egg production problems in Eastern Canada (5, 6). Necropsy examination of birds from these flocks showed high incidence of cystic left oviducts. These lesions have been previously reported in layers when infected at very young age (less than 3 weeks) (3, 8). A few IBV strains, particularly those belonging to the QX genotype, are known to induce these types of lesions (1). The initial DMV/1639 isolate was identified in a nephropathogenic infectious bronchitis outbreak in Delmarva peninsula, USA in 2011 (4). It was not until 2015 that the DMV/1639 strain was associated with infection of the reproductive tract. In addition to the IBV strain involved in infection, the age at infection is a significant factor in the induction of reproductive disease.

The aim of this study was to investigate the pathogenicity of the Canadian DMV/1639 strain for the reproductive tract of young and adult laying hens.

MATERIALS AND METHODS

Experiment 1. Forty 1-day-old SPF chicks were divided equally into two groups. The infected group was challenged oculo-nasally with 1×10^6 EID_{50} of the Canadian DMV/1639 strain in 100 µl. The control group was mock inoculated with PBS. Birds in both groups were monitored daily for clinical signs. Oropharyngeal (OP) and cloacal (CL) swabs were collected weekly from both groups. At 7 dpi, five birds from each group were euthanized. All remaining birds were euthanized at 16 weeks of age. During postmortem examination, trachea, lung, kidney, cecal tonsils, ovary, and oviduct were collected for histopathological examination and quantification of viral load by qPCR.

Experiment 2. Twenty 29-week-old SPF laying hens were divided equally into two groups. Viral/mock (dose and route) inoculation was performed as described in experiment 1. Birds in both groups were monitored daily for clinical signs and egg production for 10 dpi. OP and CL swabs were collected at 3, 5, and 10 dpi. At 5 and 10 dpi, five birds from each group were euthanized. During postmortem examination, reproductive organs (ovary and oviduct) were collected for histopathological examination and quantification of viral load by qPCR.

All statistical analyses were conducted at a 0.05 level of significance.

RESULTS

Experiment 1. Significantly higher clinical signs scores were recorded in the infected group between 3 to 7 dpi compared to the control group. Approximately 60%–70% of the infected birds shed the virus through OP and CL routes until 28 dpi and 98 dpi, respectively. The IBV genome load was quantifiable in all tissues of the infected birds collected at 7 dpi. The presence of the IBV antigen in the infected tissues was confirmed using immunofluorescence staining. At 16 weeks of age, cystic lesions of varying sizes were detected in 46% of the oviducts of the infected birds. Microscopical examination showed epithelial sloughing and mononuclear cell infiltrations in the infected ovary and oviduct in addition to cystic dilated glands in the infected oviduct. The control group remained IBV negative and showed no clinical signs, gross or microscopic lesions throughout the experiment.

Experiment 2. The infected birds were lethargic starting at 4 dpi. The egg production was between 20% to 60% in the infected group starting at 5 dpi. While no gross lesions were detected in the infected birds at 5 dpi, two birds had regressed ovary and atrophied oviduct at 10 dpi. A significant increase in the IBV genome load was detected in the infected oviduct at 10 dpi compared to at 5 dpi. Histopathological changes involved heterophilic and mononuclear cell infiltrations, sloughed epithelium, and degenerated glands in the infected oviduct. The control group remained IBV negative and had > 80% egg production throughout the experiment.

DISCUSSION

In the provinces of Ontario and Quebec, the prevalence of the DMV/1639 IBV strain in egg-laying flocks has been correlated to the low peak production (40%–77%) (7). Our findings corroborate field observations, in which infection of 1-day-old chicks with the Canadian DMV/1639 strain resulted in a significant rate of cystic formation in the oviducts of growing pullets. These chickens are usually unproductive (false layers), and affected flocks are often culled (2). The pathogenicity of the Canadian DMV/1639 strain to the reproductive tract of adult hens was also confirmed. When the chickens were challenged during peak egg production, there was a considerable decline in egg production, as well as distinctive gross and histological lesions in the reproductive tract.

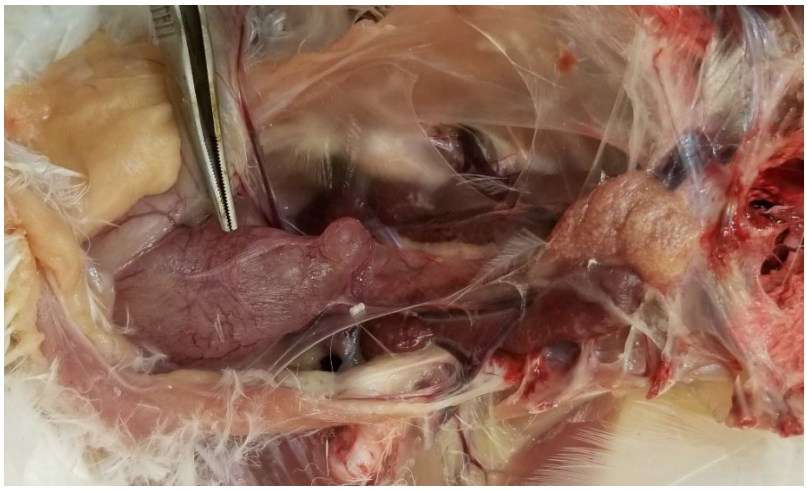
Overall, infection of young and adult chickens with the Canadian DMV/1639 strain of IBV causes substantial reproductive tract abnormalities and lowers layer reproductive performance.

(The full-length article of experiment 1 was published in *Viruses*, and a full-length article of experiment 2 will be published in *Virology*.)

REFERENCES

1. Benyeda, Z., T. Mato, T. Suveges, E. Szabo, V. Kardi, Z. Abonyi-Toth, M. Rusvai, and V. Palya. Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. Avian Pathol 38:449-456. 2009.
2. Crinion, R. A., R. A. Ball, and M. S. Hofstad. Abnormalities in laying chickens following exposure to infectious bronchitis virus at one day old. Avian Dis 15:42-48. 1971.
3. Crinion, R. A., and M. S. Hofstad. Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis 16:351-363. 1972.
4. Gelb, J., Jr., B. S. Ladman, C. R. Pope, J. M. Ruano, E. M. Brannick, D. A. Bautista, C. M. Coughlin, and L. A. Preskenis. Characterization of nephropathogenic infectious bronchitis virus DMV/1639/11 recovered from Delmarva broiler chickens in 2011. Avian Dis 57:65-70. 2013.
5. Hassan, M. S. H., D. Ojkic, C. S. Coffin, S. C. Cork, F. van der Meer, and M. F. Abdul-Careem. Delmarva (DMV/1639) Infectious Bronchitis Virus (IBV) Variants Isolated in Eastern Canada Show Evidence of Recombination. Viruses 11. 2019.
6. Parent, E., A. Gagnon-Francoeur, B. Lanthier, G. Hébert, S. Buczinski, and M. Boulianne. Diagnostic Accuracy of Ultrasonography to Detect False Layers in a Commercial Laying Flock Infected by an Infectious Bronchitis Virus Delmarva Genotype Causing Cystic Oviducts. Avian Dis 64:149-156. 2020.
7. Petrik, M. Clinical Presentation of False Layer Syndrome Caused by Infectious Bronchitis. In Proceedings of the AVMA/American Association of Avian Pathology Annual Meeting, Denver, CO, USA. 2018.
8. Zhong, Q., Y. X. Hu, J. H. Jin, Y. Zhao, J. Zhao, and G. Z. Zhang. Pathogenicity of virulent infectious bronchitis virus isolate YN on hen ovary and oviduct. Vet Microbiol 193:100-105. 2016.

Figure 1. Cystic formation in the oviduct (16 weeks of age) of a chicken infected with the Canadian DMV/1639 strain of IBV at 1-day of age.



INTESTINAL HEALTH: A PUZZLE WITH TOO MANY PIECES

R. Hauck

Department of Pathobiology and Department of Poultry Science, Auburn University

SUMMARY

The essence of broiler production is the conversion of feed into meat. In the gastro-intestinal tract (GIT), the feed is first mechanically ground and then chemically broken down into molecules that are small enough to be absorbed for further use elsewhere in the organism. Thus, maintaining intestinal health is at the same time extremely important. It is also extremely difficult, especially in broiler production with the enormous amounts of feed consumed and the necessity for optimized feed conversion.

It is an old truism that the triad of host, microorganism and environment determines the course of an infection, if infection develops into disease, and how severe the disease is. In the GIT, the most important host factors are its physiological function like nutrient transporters; tight junctions and related proteins that ensure structural integrity; as well as the local immune system, which includes mucus production. A plethora of viruses, bacteria and parasites resides in the GIT. Some of the most notable examples are diverse viruses; bacteria like Clostridia, *Escherichia coli*, *Salmonella* and *Campylobacter*; and parasites like *Eimeria* spp.; flagellated parasites *Histomonas meleagridis* and *Tetratrichomonas gallinarum*; as well as worms. The most important environmental factor for the GIT is the feed, especially its nutrient content like protein quantity and quality as well as levels of non-starch polysaccharides; the feed form including particle size; and feed additives like coccidiostats or one or several of the myriad of probiotics, prebiotics and plant products that have been tested and are on the market. Some factors that are considered important can fall into two categories, depending on the situation. *Bacillus* and *Lactobacillus* can be naturally occurring microorganisms in the GIT or can be used as probiotics. Short chain fatty acids can either be metabolites of the intestinal microbiota or be used as feed additives.

All these components interact with each other, constituting an extremely complex network. To illustrate this, a list of 22 factors, roughly the ones outlined above, was compiled and corresponding search terms for literature research were defined. These factors were translated into appropriate search terms and combined pairwise for 231 PubMed queries. Search terms for chicken-related publications and excluding review articles were added to each query.

The results of the queries were combined and the network connecting the factors was analyzed. The thickest edges in the network (Figure 1) were between *Salmonella* and *E. coli* with 1054 articles mentioning both bacteria and between coccidia and coccidiostats with 1005 articles. Searching for 21 factor pairs did not yield results. These included few unexpected combinations, like the combination of nutrient absorption and enteric viruses, but mostly combinations like coccidiostats and enteric viruses that are usually to be considered of minor relevance. The best-connected nodes in the network were the immune system with a total of 5541 connections to other nodes, *E. coli* with 4705 connections and plant products with 4543 connections. The least-connected nodes were nematodes with 330 connections, flagellate parasites with 84 connections and enteric viruses with 49 connections. The latter seemed surprising. However, much of the literature on enteric viruses in poultry revolves around finding evidence that one virus or another is a pathogen, often in epidemiological studies, sometimes after experimental infection, without considering further factors. Obviously, some factors like "plant products" were very heterogenous, while other factors like the immune system are complex systems in themselves. Furthermore, results were not further curated, so that no scientifically valid conclusions beyond the illustration of trends can be drawn from this exercise.

A path through the network connecting all 22 nodes with reasonably well demonstrated correlations further illustrates the complexity of interactions. The starting point are Rotaviruses that were positively correlated with the relative abundance of Clostridiaceae in field samples (1). Translocation of *Clostridium perfringens* out of the intestines to the liver was reduced by *Enterococcus faecium* as probiotic (2), which also reduced colonization of the ileum by *Salmonella* Typhimurium (3). *Salmonella* Enteritidis persistence in the ceca was reduced by cinnamon (4). Feed supplementation with cinnamon down-regulated expression of IL-8 (5). In contrast, IL-8 was up-regulated after infection with Avian Pathogenic *E. coli* (6), but *E. coli* counts in the ceca were reduced by yeast as feed additive (7), which also increased *Lactobacillus* populations in ileum and ceca

(8) as did higher levels of methionine in the feed (9). Lowering crude protein levels in the feed increased the concentration of several short-chain fatty acids in the ceca (10). The relative abundance of butyrate- and lactic acid-producing bacteria in the ceca were increased by monensin (11). The interactions between coccidiostats and *Eimeria* spp. are well documented and deserve no further comment. Co-infection with *Eimeria tenella* increased levels of cecal *Campylobacter* (12). In turn, challenge with *Campylobacter* changed expression of various transporter genes in the small intestines (13). Similarly, expression of glucose and peptide transporters was up-regulated by *Bacillus* species as feed additives (14), and various *Bacillus pumilus* strains up-regulated expression of mucin (15). The number of mucus-producing goblet cells was reduced when the birds received a pelleted diet (16). There was a trend that smaller particle sizes increased expression of tight junction genes (17), but expression of tight junction genes was reduced by higher levels of non-starch polysaccharides in the feed (18). Worm burdens with *Heterakis gallinarum* were higher after experimental infection in birds fed diets with higher levels of non-starch polysaccharides (19), and *H. gallinarum* serves as a paratenic host for the flagellate parasite *H. meleagridis* (20). Within the broadly defined factor "flagellate parasites", detection of *H. meleagridis* and *T. gallinarum* negatively correlated in field samples (21).

The more granular our knowledge of the individual components and their interactions becomes, greatly helped by recent technological advances, the more difficult it gets to interpret results. In addition to the question if the correlations and interactions observed in the cited experiments are direct or indirect, uncertain reproducibility and a lack of knowledge about the biological relevance of the results makes drawing broader conclusions difficult.

Reproducibility is the expectation that results of an experiment should be the same when the experiment is repeated. This can be approximated in a scientific setting, where many parameters can be controlled or at least documented. However, experience also shows that even under experimental conditions, sometimes experiments cannot be replicated. One potential reason is the presence of factors that might change or overlie the effect of the investigated treatment, but that are beyond control and means for documentation. For this reason, it is also uncertain if treatments that have shown small effects in experiments will show the same effects consistently in the field. One method to improve the reliability of conclusions is meta-analysis of experiments. In its simplest form, meta-analysis calculates a cumulative effect size, e.g. difference between treated and untreated groups, with a 95%-confidence interval from the individual experiments using their averages, measures of variance and number of observations. The influence of additional factors on the effect sizes can be determined, while all other factors are neglected. If there is a significant effect of the treatment despite all differences in the included experiments, a broad applicability of the conclusion can be assumed. Not many meta-analyses in the field of poultry health and poultry science have been published. Kipper et al. quantified the effects of coccidia challenges on weight gain and feed intake of chickens (22), while Eckert et al. conducted a network analysis to show that vaccination of broilers against *Eimeria* spp. resulted in protection similar to the use of coccidiostats (23) and Kim et al. determined the effect of coccidia on the apparent ileal amino acid digestibility (24). A novel and to date little explored approach is the meta-analysis of the bacterial microbiota. Using this method it was shown that infection with coccidia significantly changed the microbiota, but there was little consistent influence on the detected bacteria (25).

Interpreting of changes in the intestinal microbiota is generally difficult because little is known about functions and significance of most bacterial species or operational taxonomic units that are detected. Results of the Human Genome Project indicate that the metabolic function of the microbiota of different sites is very stable even though the composition of the microbiota varies widely (26). One factor that seems to be positively correlated with health is the α-diversity of the microbiota, i.e. the number of different bacteria in one host (27).

In conclusion, today we know an astonishing wealth of details how different factors in the GIT interact, but the interpretation of the results of experiments to see the bigger picture poses significant challenges. Artificial intelligence will be a valuable tool to analyze complex results and interaction and to draw useful conclusions.

REFERENCES

1. Day JM, Oakley BB, Seal BS, Zsak L. Comparative analysis of the intestinal bacterial and RNA viral communities from sentinel birds placed on selected broiler chicken farms. PLoS One 102015. doi:10.1371/journal.pone.0117210

2. Wu Y, Zhen W, Geng Y, Wang Z, Guo Y. Pretreatment with probiotic *Enterococcus faecium* NCIMB 11181 ameliorates necrotic enteritis-induced intestinal barrier injury in broiler chickens. Sci Rep 9:10256; 2019. doi:10.1038/s41598-019-46578-x

3. Zhang H, Wang M, Jia J, Zhao J, Radebe SM, Yu Q. The protective effect of *E. faecium* on *S.* Typhimurium infection induced damage to intestinal

mucosa. Front Vet Sci 8:740424; 2021. doi:10.3389/fvets.2021.740424

4. Upadhyaya I, Upadhyay A, Kollanoor-Johny A, Mooyottu S, Baskaran SA, Yin H-B, Schreiber DT, Khan MI, Darre MJ, Curtis PA, et al. In-feed supplementation of trans-cinnamaldehyde reduces layer-chicken egg-borne transmission of *Salmonella enterica* serovar Enteritidis. Appl Environ Microbiol 81:2985–2994; 2015. doi:10.1128/AEM.03809-14

5. Guo S, Cheng Q, Li Y, Duan R, Hou Y, Yi D, Ding B. Effects of dietary coated-oleum cinnamomi supplementation on the immunity and intestinal integrity of broiler chickens. Anim Sci J 89:1581–1590; 2018. doi:10.1111/asj.13094

6. Elnagar R, Elkenany R, Younis G. Interleukin gene expression in broiler chickens infected by different *Escherichia coli* serotypes. Vet World 14:2727–2734; 2021. doi:10.14202/vetworld.2021.2727-2734

7. Khalid AH, Ullah KS, Naveed S, Latif F, Pasha TN, Hussain I, Qaisrani SN. Effects of spray dried yeast (*Saccharomyces cerevisiae*) on growth performance and carcass characteristics, gut health, cecal microbiota profile and apparent ileal digestibility of protein, amino acids and energy in broilers. Trop Anim Health Prod 53:252; 2021. doi:10.1007/s11250-021-02684-5

8. Zhen W, Liu Y, Shao Y, Ma Y, Wu Y, Guo F, Abbas W, Guo Y, Wang Z. Yeast β-Glucan altered intestinal microbiome and metabolome in older hens. Front Microbiol 12:766878; 2021. doi:10.3389/fmicb.2021.766878

9. Dahiya JP, Hoehler D, Van Kessel AG, Drew MD. Effect of different dietary methionine sources on intestinal microbial populations in broiler chickens. Poult Sci 86:2358–2366; 2007. doi:10.3382/ps.2007-00133

10. Gharib-Naseri K, Dorigam JCP, Doranalli K, Morgan N, Swick RA, Choct M, Wu S-B. *Bacillus amyloliquefaciens* CECT 5940 improves performance and gut function in broilers fed different levels of protein and/or under necrotic enteritis challenge. Anim Nutr 7:185–197; 2021. doi:10.1016/j.aninu.2020.05.004

11. Robinson K, Becker S, Xiao Y, Lyu W, Yang Q, Zhu H, Yang H, Zhao J, Zhang G. Differential impact of subtherapeutic antibiotics and ionophores on intestinal microbiota of broilers. Microorganisms 7:E282; 2019. doi:10.3390/microorganisms7090282

12. Macdonald SE, van Diemen PM, Martineau H, Stevens MP, Tomley FM, Stabler RA, Blake DP. Impact of *Eimeria tenella* coinfection on *Campylobacter jejuni* colonization of the chicken. Infect Immun 872019. doi:10.1128/IAI.00772-18

13. Garcia JS, Byrd JA, Wong EA. Expression of nutrient transporters and host defense peptides in *Campylobacter* challenged broilers. Poult Sci 97:3671–3680; 2018. doi:10.3382/ps/pey228

14. Biswas A, Dev K, Tyagi PK, Mandal A. The effect of multi-strain probiotics as feed additives on performance, immunity, expression of nutrient transporter genes and gut morphometry in broiler chickens. Anim Biosci 35:64–74; 2022. doi:10.5713/ab.20.0749

15. Bilal M, Si W, Barbe F, Chevaux E, Sienkiewicz O, Zhao X. Effects of novel probiotic strains of *Bacillus pumilus* and *Bacillus subtilis* on production, gut health, and immunity of broiler chickens raised under suboptimal conditions. Poult Sci 100:100871; 2020. doi:10.1016/j.psj.2020.11.048

16. Mohammadi Ghasem Abadi MH, Moravej H, Shivazad M, Karimi Torshizi MA, Kim WK. Effects of feed form and particle size, and pellet binder on performance, digestive tract parameters, intestinal morphology, and cecal microflora populations in broilers. Poult Sci 98:1432–1440; 2019. doi:10.3382/ps/pey488

17. Barzegar M, Zaghari M, Zhandi M, Sadeghi M. Effects of zinc dosage and particle size on gut morphology, tight junctions and TNF-α expression in broiler breeder hens. J Anim Phys AnimNutri; 2021. doi:10.1111/jpn.13638

18. Santos RR, Ooosterveer-van der Doelen MAM, Tersteeg-Zijderveld MHG, Molist F, Gehring R. Induction of gut leakage in young broiler chickens fed a diet with low rye inclusion. Heliyon 7:e08547; 2021. doi:10.1016/j.heliyon.2021.e08547

19. Daş G, Abel H, Rautenschlein S, Humburg J, Schwarz A, Breves G, Gauly M. Effects of dietary non-starch polysaccharides on establishment and fecundity of *Heterakis gallinarum* in grower layers. Vet Parasitol 178:121-128; 2011. doi:10.1016/j.vetpar.2010.12.027

20. Graybill HW, Smith T. Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. J Exp Med 31:647–655; 1920. doi:10.1084/jem.31.5.647

21. Hauck R, Balczulat S, Hafez HM. Detection of DNA of *Histomonas meleagridis* and *Tetratrichomonas gallinarum* in German poultry flocks between 2004 and 2008. Avian Dis 54:1021–1025; 2010. doi:10.1637/9261-012910-Reg.1

22. Kipper M, Andretta I, Lehnen CR, Lovatto PA, Monteiro SG. Meta-analysis of the performance variation in broilers experimentally challenged by *Eimeria* spp. Vet Parasitol 196:77–84; 2013. doi:10.1016/j.vetpar.2013.01.013

23. Eckert J, Carrisosa M, Hauck R. Network meta-analysis comparing the effectiveness of anticoccidial drugs and anticoccidial vaccination in broiler chickens. Vet Parasitol 291:109387; 2021. doi:10.1016/j.vetpar.2021.109387

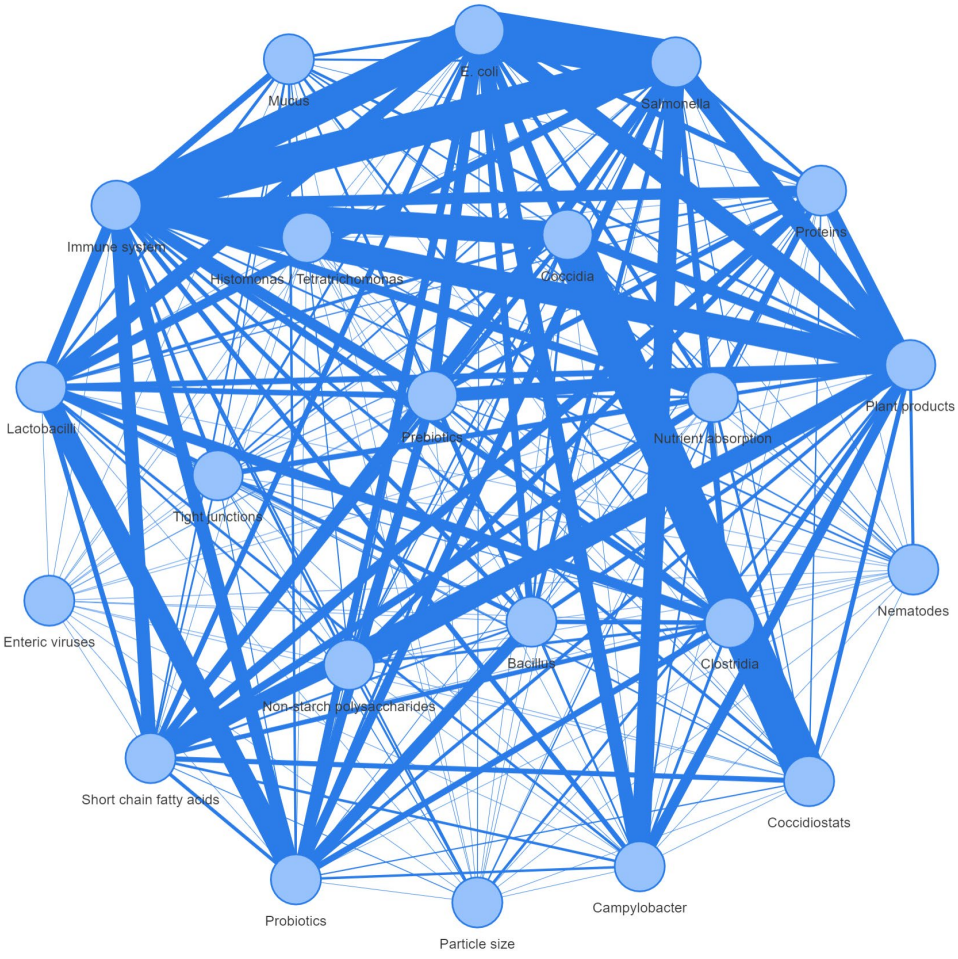
24. Kim E, Létourneau-Montminy M-P, Lambert W, Chalvon-Demersay T, Kiarie EG. Centennial Review: A meta-analysis of the significance of *Eimeria* infection on apparent ileal amino acid digestibility in broiler chickens. Poult Sci 101:101625; 2022. doi:10.1016/j.psj.2021.101625

25. Pietruska A, Hauck R. A meta-analysis: microbiome of chickens infected with coccidia. In: Abstracts of the International Poultry Scientific Forum. p. 51; 2022.

26. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG, Fulton RS, et al. Structure, function and diversity of the healthy human microbiome. Nature 486:207–214; 2012. doi:10.1038/nature11234

27. Mosca A, Leclerc M, Hugot JP. Gut microbiota diversity and human diseases: should we reintroduce key predators in our ecosystem? Front Microbiol 7:455; 2016. doi:10.3389/fmicb.2016.00455

Figure 1. A network of scientific articles connecting important factors that determine intestinal health in chickens shows the complex interactions.



COMPARISON OF THE FIELD APPLICATION OF A COMMERCIAL COCCIDIOSIS VACCINE IN BROILERS WITH BIO-SHUTTLE PROGRAM

R. Jacob

Boehringer Ingelheim Animal Health USA Inc., Duluth GA, 30096

SUMMARY

Bio-shuttle programs which start with hatchery vaccination followed by using an in-feed anticoccidial is becoming increasingly popular in the field. Field application of coccidiosis vaccine during the first few days in the broiler house has also been proven effective in controlling clinical coccidiosis. The present study compares the effect of a field application of a live coccidiosis vaccine with a bio-shuttle program in commercial broiler complexes in the USA. Data showed that the use of hatchery application of the vaccine alone, or together with field application in the first few days showed statistically significant improvement in the average weight and average daily gain in broilers. The selection of anticoccidial used in a bio-shuttle program also had an impact on broiler performance.

INTRODUCTION

Coccidiosis, caused by the protozoa *Eimeria,* is a disease of high economic significance in commercial chickens worldwide. Control strategies against coccidiosis are based on in-feed anticoccidial drugs and/or live coccidiosis vaccines (1). Most of the commercially available coccidiosis vaccines in the USA for broilers are based on live sporulated oocysts of three *Eimeria* species: *E. acervulina, E. maxima,* and *E. tenella.* Mass administration of the coccidiosis vaccine and its subsequent oral ingestion by the chicks is critical to the success of any live coccidiosis vaccination program (2). This is accomplished at the hatchery because of a more controlled environment. However, most of the broiler companies do not solely depend on hatchery vaccination alone for the successful control of coccidiosis. Bio-shuttle programs combine the administration of a vaccine with feeding of anticoccidial ionophores or chemicals in the grower feed, or grower and finisher feed. In such programs, the timing and dosage of the anticoccidial used in the operation determine the efficacy the coccidiosis cycling. Anticoccidials should not be added to the feed too early to minimize the risk of interfering with immunity development from the vaccines. Field application of coccidiosis vaccine during the first few days in the broiler house has also been proven as effective in the control of clinical coccidiosis (3). This study compares the effect of a commercially available coccidiosis vaccine (Hatchpak Cocci III, Boehringer Ingelheim Animal Health) when used alone in the hatchery, combined with field application or in a bio-shuttle program in commercial broilers raised in the USA.

MATERIALS AND METHODS

Location. Two different commercial broiler complexes (Complex A & Complex B) in the southern part of the USA.

Birds. Over 60 million broilers placed between March – October 2021 were represented in this study.

Coccidiosis control programs. Complex A, with farms located in the Southern states of the USA started a Bio-shuttle program with Hatchpak Cocci III (hatchery spray) and Zoelene (in-feed, grower feed) during the week of April 4, 2021. On June 6th they stopped the bio-shuttle program and started field application of the vaccine along with hatchery application. Starting August 1^{st,} they've stopped field application and only used a single dose of the vaccine applied in the hatchery for coccidiosis control in their broilers.

Complex B, also with farms located in the Southern states of the USA, started a bio-shuttle program with Hatchpak Cocci III (hatchery spray) with monensin (in-feed, grower feed) on March 7, 2021. On June 6th they've changed to a different bio-shuttle program with Hatchpak Cocci III (hatchery spray) with salinomycin (in-feed, grower feed).

Parameters measured. Coccidia cycling patterns and other performance indicators normally used by broiler integrators in the USA including livability, age, average body weight, average daily weight gain, and adjusted feed conversion. The performance parameters were analyzed by ANOVA using the procedures of the General Linear Models of SAS 9.4. Significant interactions were further analyzed using Tukey's least-square means comparison.

Sampling schedule. Weekly performance data was collected from the complexes. Coccidia cycling patterns were monitored by field posting sessions conducted every four to six weeks.

RESULTS AND DISCUSSION

Results from the current study indicated statistically significant improvement in average weight and average daily gain in broilers receiving the vaccine alone or vaccine combined with field application, compared to the bio-shuttle program in Complex A (Table 1). The adjusted feed conversion rate was lowest for the broilers receiving a single dose of vaccine in the hatchery, which is significantly lower than the bio-shuttle program. Coccidia cycling pattern reveals mild and early cycling for the vaccine-only program compared to the other two programs used by the complex.

No field application was used as a coccidia control program in complex B. They stayed on a bio-shuttle program during the entire period, however, switched to a different program during the second rotation. Average weight, average daily gain, and adjusted feed conversion were statistically better when the vaccine was combined with salinomycin as an in-feed program in the grower feed compared to the use of monensin.

REFERENCES

1. Cervantes HM., McDougald LR, Jenkins MC. Coccidiosis. In: Swayne D, editor. Diseases of poultry, 14th ed. Hoboken (NJ): WileyBlackwell. p. 1193–1217; 2020.

2. Schaeffer J, Ritter D, Jenkins M, Ameiss K. Necrotic Enteritis and factors critical for successful coccidiosis vaccination outcomes. In: Frame, DD, editor. Proceedings of the 65th Western Poultry Disease Conference; 2016 Apr 24–27; Vancouver (Canada). Jacksonville (FL): American Association of Avian Pathologists. p. 228-230; 2016.

3. McCarty J. The impact of a coccidiosis vaccine field boost. In: Frame, DD, editor. Proceedings of the 66th Western Poultry Disease Conference; 2017 Mar 20-22; Sacramento (California). Jacksonville (FL): American Association of Avian Pathologists. p. 125. 2017.

Table 1. Performance differences between coccidiosis control programs in commercial broilers. Means within each column with no common superscripts differed significantly (P<0.05).

	Program	Parameters measured				
		Livability (%)	**Age (days)**	**Average weight (lbs)**	**Average daily gain (lbs/day)**	**Feed conversion (Adjusted)**
Complex A	Bio-shuttle Vaccine + Zoelene	95.60	38.94^{b}	4.32^{b}	0.1109^{b}	1.594^{b}
	Vaccine + Field application	95.98	40.59^{a}	4.79^{a}	0.1180^{a}	1.572^{ab}
	Vaccine only	95.47	40.39^{a}	4.75^{a}	0.1176^{a}	1.566^{a}
Complex B	Bio-shuttle 1 Vaccine+ Monensin	93.91	34.92	3.73^{y}	0.1069^{y}	1.645^{y}
	Bio-shuttle 2 Vaccine+Salinomycin	94.42	35.33	3.91^{x}	0.1108^{x}	1.617^{x}

ASSESSMENT OF THE IMPACT OF AN Ɛ-POLYLYSINE PRODUCT ON HATCHABILITY, BACTERIAL LOADS AND SEVEN-DAY PERFORMANCE IN BROILER CHICKS FOLLOWING *IN OVO* INOCULATION

L. Johnson[A], A. Kiess[C], C. Castañeda[B], O. Gutierrez[A], and D. Smith[A]

[A]Huvepharma, Inc., 525 Westpark Dr, Suite 230, Peachtree City, GA 30269
[B]Department of Poultry Science, Mississippi State University, MS 39762
[C]Department of Poultry Science, North Carolina State University, Raleigh, NC 27695

SUMMARY

As public concern over the use of antibiotics increases, alternatives must continue to be investigated. ε-Polylysine, a homo-polymer of L-lysine with antimicrobial activity, may be such an alternative. In this experiment, fertile broiler hatching eggs were evenly distributed among six treatment groups and inoculated *in ovo* on day 18 of incubation. At hatch, hatchability and average chick weight were determined. All male chicks were transported to a battery facility to evaluate microbiological and growth performance attributes over a seven-day period of time. The results of this study indicate that the inclusion of ε-Polylysine did not negatively impact hatchability or live performance when compared to the control groups. Though microbiological data was collected on day 0 and day seven of the grow-out, bacterial counts for all treatments were too low to be included in the analysis. Therefore, additional studies are warranted to determine the efficacy of ε-Polylysine in a commercial broiler environment.

INTRODUCTION

Increasing concerns over antimicrobial resistance in poultry has led to a reduction in antibiotic use in hatcheries and feed. Consequently, antimicrobial alternatives must maintain animal health as well as production efficiency (1). ε-Polylysine may be such an alternative with positive outcomes when delivered to poultry. ε-Polylysine, an antimicrobial peptide synthesized in aerobic bacterial fermentation, is commonly used as a food preservative (2). It has been reported to be effective against a wide variety of microorganisms including yeasts, fungi and bacteria (3, 4). In an *in vitro* study, it was demonstrated that when the concentration of ε-Polylysine exceeds 0.45%, the presence of several serotypes of *Salmonella* is reduced (5). Therefore, this study was designed to evaluate the impact of ε-Polylysine on hatchability, bacterial loads and live performance in broilers.

MATERIALS AND METHODS

Hatchery and treatment groups. Initially, 3,240 fertilized eggs were purchased from a commercial breeder flock composed of 44-week-old Ross 708 hens with a reported fertility of 84%. All eggs were weighed and the average egg weight was identified. All eggs were then sorted by weight, assigned to one of six treatments and placed into one of three NatureForm (Model NMC-1080, Pas Reform North America, Jacksonville, FL.) setters. Each setter had 6 turning levels, which held 6 flats of eggs (30 eggs/flat) per level. A flat of eggs that represents each treatment was randomly assigned to each turning level of each setter. On day 10, eggs were candled and any cracked, contaminated, or early dead embryos were removed. On day 18, eggs from each treatment were removed from the setter and commercially *in ovo* inoculated with the appropriate treatment; Marek's vaccine alone (50µl (Negative Control)); Marek's vaccine + Gentamicin (50µl/0.1mg (Positive Control)); Marek's vaccine + ε-Polylysine (50µl/0.05%); Marek's vaccine + ε-Polylysine (50µl/0.1%); Marek's vaccine + ε-Polylysine (50µl/0.2%); Marek's vaccine + ε-Polylysine (50µl/0.4%). After inoculation, 180 eggs per treatment (6 flats/2 from each setter from different levels) were transferred to one of 18 individual GQF (GQF Manufacturing Company Inc., Savannah, GA.) incubators for hatch (6 treatments X 3 incubators/treatment = 18 incubators).

Broiler grow-out. On day 21, all hatched chicks were counted and weighed. Eggs that did not hatch were broken out for hatch residue. As individual chicks were counted, they were also feather sexed and all male chicks were moved into a battery facility and re-weighed. At the battery facility, 15 male broilers were placed into a battery cage (6 treatments at the hatchery X 12 replicate pens = 72 battery cages; 72

battery cages X 15 male broilers per battery cage = 1,080 male broilers total). Each battery cage was equipped with a feeder and 2 nipple drinkers to provide the broilers with feed and water *ad libitum*.

Performance measurements and microbial sampling. On day 0 (12 h after hatch) and day seven, each broiler was weighed individually along with the pen feeder to obtain live weight gain, cage intake, feed intake and adjusted feed conversion ratio (FCR). On day 0, three broilers per cage were humanely euthanized, yolk sacs were removed aseptically, pooled into a sterile plastic sampling bag, and then placed on ice until transported back to the laboratory for microbiological analysis. Microbiological analysis consisted of quantification of all gram-positive bacteria, *Escherichia coli*, *Pseudomonas*, *Salmonella*, *Staphylococcus* and *Enterococcus*. On day seven, three broilers per cage were euthanized and cecal tonsils were removed aseptically. The cecal tonsils were pooled into a sterile plastic sampling bag, placed on ice, transported back to the laboratory and analyzed for *Salmonella* concentrations. All animals were treated in accordance with the guide for the care and use of agricultural animals for research and teaching.

Data analysis. Hatch of transfer data was collected throughout the 21-day hatching process. Variables of interest included hatch of transfer, infertile eggs, early dead embryos, mid dead embryos, late dead embryos, pipped embryos, cracked eggs, contaminated embryos and average chick weight. Live performance data was collected from day 0 to day seven of the grow-out. Variables measured included live weight gain, end weight gain, pen intake, feed intake, mortality and FCR adjusted for mortality. Hatchability data were analyzed using a completely randomized design. Growth performance data were analyzed using a randomized complete block design in SAS for Windows version 9.4 (SAS Institute, Cary NC.) at a significance level of 0.05.

RESULTS

There were no significant differences detected for any of the hatchability (Table 2) or live performance (Table 3) variables evaluated across all treatment groups. However, though not statistically significant, the highest concentration of ε-Polylysine (Treatment group 6) improved hatch by almost 2% compared to the negative control and all ε-Polylysine treatments were higher than the negative control. Additionally, Treatments 3 and 4 reduced the FCR by approximately 0.01 and the live weight gain for Treatment 4 was approximately 10 grams heavier than the negative control.

Naturally-occurring bacteria associated with fertile broiler hatching eggs were evaluated. However, the counts obtained for all treatments were non-detectable and no statistical analysis could be conducted. For day 0, the number of colonies on the media plates was too low for data entry (less than 30 colonies). A few random pens scattered among treatments did have countable plates (greater than 30 colonies), but the number of those plates was not high enough to support appropriate statistical analysis. On day seven, cecal content and tissues were placed in nutrient broth for enrichment of *Salmonella* and changes in the broth indicated that bacterial growth had occurred. However, when the organisms in the broth were streaked onto specific selective agars (XLT4 and Brilliant Green) for *Salmonella* confirmation, the results demonstrated that the growth represented *Escherichia coli* and not *Salmonella*.

DISCUSSION

Though there were no significant differences between treatment groups for the hatchability variables, these results demonstrate that ε-Polylysine can be safely administered *in ovo* at a concentration as high as 0.4% without disrupting normal hatchery efficiencies. Also, the increase in hatchability across all treatment groups when compared to the negative control group seems to be primarily driven by a reduction in late dead embryos. Even small increases in hatchability can have a big impact in commercial poultry hatcheries.

For the live performance results, it was expected that Treatment 2 (Gentamicin), would provide an advantage in performance over the other treatment groups. However, this trial was conducted in a battery cage facility without all of the normal environmental stressors and it was only conducted for seven days. Due to these factors, the advantages of an antibiotic and/or ε-Polylysine treatment may have been masked. Additionally, there could be other characteristics that were improved but not measured during this trial, such as immune status parameters.

Although microbiological data was collected on day 0 and day seven of the grow-out, bacterial counts for all treatments were too low to be included in the analysis. The eggs received from this trial were likely higher quality fertile eggs due to the fact that it was performed at a research university. In addition, the trial facilities have fewer challenges when it comes disease-causing organisms due to the volume of eggs and broilers hatched and raised compared to a commercial broiler operation. Future studies should include stressors, such as heat stress or a coccidiosis challenge, as well as a bacterial challenge such as Avian Pathogenic *Escherichia Coli* (APEC) to more closely mimic the commercial broiler hatchery and housing environments.

Due to the route of application, the compatibility of ε-Polylysine with Marek's vaccine was considered. Unfortunately, the addition of ε-Polylysine alters the vaccine solution pH to the point it would not be a viable *in ovo* antimicrobial alternative in hatcheries when administered in conjunction with Marek's vaccine. Other methods of application may be considered in future studies, including in feed, on table eggs or in different delivery vehicles (i.e. gel).

REFERENCES

1. Tabler T, Zhai W, Wells J, Moon J. Antibiotic resistance, alternatives, and the U.S. Poultry Industry. Mississippi State University Extension Service. 2021 [accessed 2022 Feb 2]. http://extension.msstate.edu/publications/antibiotic-resistance-alternatives-and-the-us-poultry-industry

2. Hiraki J, Ichikawa T, Ninomiya S-ichi, Seki H, Uohama K, Seki H, Kimura S, Yanagimoto Y, Barnett JW. Use of ADME studies to confirm the safety of ε-polylysine as a preservative in food. Regulatory Toxicology and Pharmacology. 2003 Apr 11 [accessed 2022 Feb 2]. https://www.sciencedirect.com/science/article/abs/pii/S0273230003000291?via%3Dihub

3. Shaoyong W, Li Q, Ren Z-qiang, Wei C-sheng, Chu G-yan, Dong W-zi, Yang G-she, Pang W-jun. Evaluation of ε-polylysine as antimicrobial alternative for liquid-stored boar semen. Theriogenology. 2019 Mar 12 [accessed 2022 Feb 2]. https://www.sciencedirect.com/science/article/abs/pii/S0093691X18310501

4. Ye R, Xu H, Wan C, Peng S, Wang L, Xu H, Aguilar ZP, Xiong Y, Zeng Z, Wei H. Antibacterial activity and mechanism of action of ε-poly-L-lysine. Biochemical and Biophysical Research Communications. 2013 Aug 9 [accessed 2022 Feb 2]. https://www.sciencedirect.com/science/article/abs/pii/S0006291X1301317X?via%3Dihub

5. Duong T, Froebel L. Characterization of *Salmonella* inhibition by ε-polylysine using growth curve analysis. [Poster] International Poultry Scientific Forum. 2019 Jan. Atlanta, GA.

Table 1. Treatment groups.

Treatments #	Inoculated *in ovo* product	Concentration (µl/mg/%)
1	Marek's vaccine (MV)	50 µl
2	MV + Gentamicin	50 µl/0.1 mg
3	MV + ε-Polylysine (1)	50 µl/0.05%
4	MV + ε-Polylysine (2)	50 µl/0.1%
5	MV + ε-Polylysine (3)	50 µl/0.2%
6	MV + ε-Polylysine (4)	50 µl/0.4%

Table 2. Hatchability results.

	Trt 1	Trt 2	Trt 3	Trt 4	Trt 5	Trt 6	P value	SEM	N
Hatch of Transfer (%)	89.58	90.1	89.7	89.7	90.9	91.7	0.85	1.36	3
Infertile (%)	0	0	0.28	0	0.19	0.48	0.3	0.17	3
Early Dead (%)	0	0.25	0	0	0.2	0.24	0.7	0.16	3
Mid Dead (%)	0.2	0	0	0.23	0.21	0.21	0.84	0.18	3
Late Dead (%)	5.29	6.55	7.77	6.44	4.86	4.39	0.11	0.82	3
Pipped (%)	4.3	3.13	2.7	3.65	3.6	3	0.95	1.51	3
Cracked (%)	0	0	0	0	0	0	NA	NA	3
Contaminated (%)	0.63	0	0	0	0	0	0.46	0.26	3
Avg. Chick Weight (g)	46.5	46.2	46.2	46.1	46.6	45.9	0.64	0.32	18

Table 3. Live performance results.

	Trt 1	Trt 2	Trt 3	Trt 4	Trt 5	Trt 6	P value	SEM	N
Live Wt. Gain/Bird (kg)	0.112	0.119	0.113	0.123	0.115	0.111	0.14	0.004	12
End Wt. Gain/Bird (kg)	0.159	0.165	0.159	0.17	0.161	0.157	0.19	0.004	12
Pen Intake (kg)	1.502	1.615	1.517	1.538	1.581	1.573	0.58	0.05	12
Feed Intake/Bird (kg)	0.17	0.183	0.169	0.18	0.176	0.175	0.33	0.005	12
Mortality (%)	0	0.61	0	1.21	0	0.56	0.39	0.48	12
FCR (kg/kg)	2.189	2.112	2.079	2.056	2.157	2.184	0.65	0.005	12

RE-DEFINING THE AVIAN PATHOGENIC *ESCHERICHIA COLI* (APEC) PATHOTYPE

T. Johnson[A,B], C. Flores-Figueroa[B], J. Munoz-Aguayo[B], E. Miller[A], M. Kromm[C], K. Schaefbauer[C], B. Wileman[D], and K. Fransen[D]

[A]University of Minnesota, Department of Veterinary and Biomedical Sciences, Saint Paul, MN 55108
[B]University of Minnesota, Mid-Central Research and Outreach Center, Willmar, MN 56201
[C]Jennie-O Turkey Store, Willmar, MN 56201
[D]Select Genetics, Willmar, MN 56201

SUMMARY

Avian pathogenic *Escherichia coli* (APEC) belong to a broader pathotype known as extraintestinal pathogenic *E. coli*, and APEC is the causative agent of colibacillosis affecting all production bird types. While much work has been put forth towards defining the APEC pathotype in broilers and layer chickens, recent evidence suggests that current screening strategies may not effectively identify true APEC. Here, we assess more than 3,800 genomes from commercial turkey and broiler production, including both clinical and cecal isolates. Using a pangenomic approach, coupled with rapid virulence screens, we re-define the APEC pathotype with an emphasis on the identification of high-risk APEC clones. This information changes the way producers should view the APEC pathotype, and corresponding control strategies.

MATERIALS AND METHODS

Samples of convenience were submitted from recently deceased or moribund turkeys displaying classical signs of colibacillosis. A total of 396 isolates were collected. Isolates were collected from a total of seven major turkey producing companies in the United States, across at least nine different states. At least 155 different farm complexes were represented in the isolate collection. Isolates from both breeders and commercial birds were included. Ages ranged from day-of-hatch through 55 weeks of age. DNA sequencing was performed using 2x300-bp dual-index runs on an Illumina MiSeq at the University of Minnesota Mid-Central Research and Outreach Center. A search of Enterobase (July 2021) was then conducted for all available raw sequencing data of U.S. poultry-source isolates annotated as *Escherichia coli*. Raw sequencing reads of all identified isolates (N = 1469 for turkey meat, N = 562 for live turkey, N = 744 for chicken meat, N = 525 for live chicken) were downloaded from the SRA using the SRA Toolkit (v2.8.2), and assembled using SPAdes. Assemblies were used to screen for predicted serotype, sequence type, plasmid possession, *fimH* type, Clermont phylogenetic type, and APEC virulence factors. From this, a subset of strains was used in virulence screens. Chicken embryo lethality assays were performed using 12-day embryonated eggs inoculated with 500 colony forming units of the strain of interest. Turkey embryo lethality assays were performed using 15-day embryonated eggs inoculated with 1,000 colony forming units of the strain of interest. For each strain tested, two biological replicates were performed with n=12 eggs per replicate.

Based on the results of genomic analyses and virulence screens, five sequence types were included in the development of a revised APEC typing scheme. The entire turkey isolate collection (n=2,472 genomes) was grouped based on ST type, and pangenomic analyses were conducted for each ST on interest versus all other isolates using Roary and Scoary. From this, distinguishing gene markers were sought for each ST type. PCR primers for these gene markers were designed. The revised panel also included two markers of the APEC plasmid PAI previously included in a published scheme (1), and an O78 serogroup-specific marker previously described (2). The panel was validated by screening a subset of strains from this study (n=50) to confirm expected results based upon genomic analyses.

RESULTS

Using 396 clinical isolates from diseased commercial turkeys, we found that clinical *E. coli* from commercial turkeys are dominated by a subset of clonal groups, irrespective of geographical location or company (Figure 1). These clonal groups included sequence types ST23, ST117, ST131, ST355, and ST428. This differed from dominant cecal *E. coli* from turkeys, which were dominated by ST10 and ST58, among others. When examining possession of the APEC plasmid, which has been previously designated as a key trait of the APEC pathotype, clinical and cecal *E. coli* from commercial turkeys both possess the

APEC plasmid pathogenicity-associated island (PAI) at high proportions (Figure 2). Among turkey clinical isolates, genes within the region previously described as the "conserved virulence region" (Johnson *et al*., 2006) were found at a rate of 72-96%. In turkey cecal isolates, genes within this region were found at a rate of 45-85%. However, the genomic backgrounds of turkey clinical *E. coli* and turkey cecal *E. coli* were substantially different. The dominant phylotype among turkey clinical isolates was B2 (46%), whereas only 6% of turkey cecal isolates belonged to the B2 phylogroup. Conversely, turkey cecal isolates belonged primarily to phylotypes B1 (41%) and A (29%), compared to 8% and 2% for turkey clinical isolates, respectively. Additionally, phylotype C was overrepresented by turkey clinical isolates (19%) compared to turkey cecal isolates (2%).

A comparison of turkey clinical versus turkey cecal isolates was performed using pangenome analyses (Roary) and Scoary to identify genes significantly higher in turkey clinical populations. No clearly defining genomic traits were identified between turkey clinical and turkey cecal isolates. A total of 401 genes were identified that were 1) present in >50% turkey clinical isolates, 2) present in <50% turkey cecal isolates, and 3) had an adjusted $P < 0.05$. This approach did identify genes previously examined using different approaches falling within these prevalence ranges, including *eitABCD*, *iutA*, *iucABCD*, and *tsh*. Several additional gene clusters of interest were identified as significantly more prevalent in Turkey clinical versus Turkey cecal. These included *ccdA* and *ccdB*, a type II toxin-antitoxin system (65-67% versus 20%, OR = 7.4-8); a putative ABC transporter system (71% versus 28%, OR = 6.1-6.3); a type VI secretion system (54-55% versus 8-11%, OR = 9.0-13.1); a capsular biosynthesis cluster (57% versus 12-13%, OR = 8.7-10.2); a putative iron transport system (58-68% versus 17-21%, OR = 6.9-7.8); and a putative sugar utilization and phosphotransferase system (59-61% versus 17-18%, OR = 6.8-7.4). However, no genes were identified which were >90% present among turkey clinical isolates, and many of the genes found at >75% among turkey clinical isolates were actually allelic variants of gene systems generally conserved among *E. coli*. Furthermore, no genes were identified which were clearly dominant among clinical isolates and rare among cecal isolates, suggesting that a set of genes absolutely differentiating these two populations does not exist.

Using a similar approach, we examined chicken cecal and chicken retail database isolates for presence of APEC plasmid PAI genes and Clermont phylogenetic type. On average, APEC plasmid PAI genes were approximately 30% lower in chicken cecal versus turkey cecal isolates. For example, the conserved region of the APEC plasmid PAI (*etsABC*, *hlyF*, *iroBCDEN*, *iss*, *ompTp*, and *sitABCD*) ranged from 29-60% prevalence in chicken cecal isolates, versus 64-85% prevalence in turkey cecal isolates. Chicken retail isolates possessed the conserved region of the APEC plasmid PAI at slightly lower prevalence than Turkey retail isolates (range 61-80% versus 76-91%, respectively). Phylogenetic types of chicken cecal and chicken retail mostly mimicked those of turkey cecal and turkey retail isolates. These results indicate that the APEC plasmid is found at lower prevalence among chicken cecal isolates compared to turkey cecal isolates, but still at rates higher than previously reported.

Dominant sequence types from clinical versus cecal populations were identified, and representative strains were selected for turkey and chicken embryo lethality assays. Two themes emerged from these results. First, isolates from dominant clinical STs were clearly more virulent towards embryos than isolates from dominant cecal STs. A second theme that emerged was that there were patterns within STs between embryo lethality and APEC plasmid PAI gene content. For example, within ST23 the isolate with only 7 APEC plasmid PAI genes was less lethal towards turkey embryos than the isolate with 37 APEC plasmid PAI genes (66.7 versus 91.7%), yet it was still among the most lethal strains tested even without the APEC plasmid PAI genes. Within ST117, turkey embryo lethality mostly correlated with APEC plasmid PAI gene content for the four isolates examined (14-42 genes ranging in lethality from 4.2-54.2%). The most lethal isolates in both ELA models were strains from ST23 and ST131. In general, isolates from the B2 phylogenetic background were more lethal towards turkey embryos than other backgrounds, but this was confounded in some part by number of isolates examined and APEC plasmid PAI gene content.

From these data, a nine-target revised APEC pathotyping scheme was developed. This scheme targets the sequence types ST23, ST117, ST131, ST355, and ST428; the O78 gene cluster; and two conserved APEC plasmid genes, *hlyF* and *ompTp*. Additionally, a computational tool has been developed to screen assembled genomes for this scheme. The scheme defines high-risk APEC with possession of one of the five STs (or O78 gene cluster) plus the presence of the two APEC plasmid genes. The multiplex PCR assay was validated using a set of 50 clinical and 50 cecal isolates already sequenced in this study, and performed as expected.

DISCUSSION

This study was prompted by recent reports indicating that a commonly used 5-gene APEC typing scheme may not discriminate virulence potential, based upon the observation that high proportions of gastrointestinal-source isolates possessed APEC plasmid PAI genes (4). The definition of the APEC pathotype has been a subject of debate for many years. Recently, work by Mageiros *et al*. used genome-wide association studies in an effort to revisit this question (4). Their approach utilized broiler clinical versus "asymptomatic" isolates and determined that, as expected, clinical isolates were identified across a wide range of *E. coli* lineages. One conclusion from this study was that the APEC pathotype is complex because it relies on combinations of fitness- versus virulence-associated traits, and likely the blend of plasmid-associated traits in multiple, optimal chromosomal backgrounds. In this study, we took a different approach based on the observation that few genomic differences existed between clinical versus cecal isolates within a respective ST type. Instead, we sought to:

1) identify STs that were dominant within clinical isolates versus within cecal isolates.
2) determine the virulence potential for each of those dominant STs in a simple embryo lethality assay screen.
3) determine genes overall that were enriched in clinical versus cecal isolates.
4) identify gene markers of specific clinical STs of interest.

Similar to Mageiros *et al*., we found that ColV plasmid PAI genes were highly prevalent not only in clinical isolates, but also in cecal isolates. Using the previously established pentaplex PCR (predicted via WGS data) for APEC (1), the number of APEC classified as have 4 or more of the genes *iss*, *iroN*, *hlyF*, *ompTp*, and *iutA* was 81.1% versus 65.5% in clinical versus cecal, respectively. This appears to be in line with the results obtained from Mageiros *et al*. (4). Importantly, these genes and almost all of the other APEC plasmid-associated genes were still of significantly higher prevalence in clinical versus cecal populations. However, the relatively high prevalence of APEC plasmid genes in cecal isolates indicates that they may be less useful as a diagnostic tool for discriminating highly virulent APEC. The results of our embryo lethality experiments support this concept, as presence of the APEC plasmid alone in an isolate did not necessarily provide it with high virulence potential. However, we observed striking differences with respect to phylotypes of clinical versus cecal isolates. In contrast to Mageiros *et al.*, we found that clinical isolates were heavily dominated by the B2 and C phylotypes, compared to cecal isolates. This contrasts not only Mageiros *et al*., but also multiple previous studies where there were less clear distinctions between populations with respect to phylotype, and notably proportionally fewer clinical isolates belonging to the B2 phylotype (4). We currently do not know if this represents a temporal shift in APEC populations across poultry production in the US, or is simply reflective of inherent differences between true APEC from commercial turkey versus commercial broiler production. Nevertheless, it is clear from this work that specific combinations of genomic background (ST) plus APEC plasmid carriage result in highly virulent, and thus high-risk, clones.

The results of the ELA assays in turkey and chicken embryos indicate that the simple use of the presence of APEC plasmid PAI genes alone is not sufficient to fully discriminate between high risk versus lesser risk *E. coli* clones. This is supported by two lines of evidence. First, in STs such as ST58, isolates which contained a high number of APEC plasmid PAI genes (33-35) were not lethal in the ELA models. This, and our genomic screens, indicate that dominant gut strains commonly possess genes of the APEC plasmid PAI but are not particularly virulent towards birds. Second, within dominant clinical STs, isolates that would be classified as APEC solely using the 5-gene APEC plasmid PAI scheme differed in their lethality towards embryos (e.g., ST117 and ST428). With that said, our data and the data of others clearly shows that the presence of the APEC plasmid or its genes enhances virulence in certain clonal backgrounds. However, some clones are highly virulent even when they lack APEC plasmid PAI genes, such as ST23. Together, the evidence indicates that the best means to identify high-risk clones for poultry producers needs to include the presence of the APEC plasmid in combination with clone-specific markers.

Using the information gleaned from the genomic and phenotypic experiments in this study, we propose here the use of a revised approach to APEC typing in poultry. Rather than focusing solely on specific sets of virulence and fitness factors, we propose the use of markers of the APEC plasmid combined with ST- or serogroup-specific genomic background as an improved tool. The presence of the two APEC plasmid markers (*hlyF* and *ompTp*), which are among the most highly conserved of the plasmid, plus the presence of one of the clonal background-specific markers (ST23, ST117, ST131, ST335, ST428, or O78), would indicate presence of a high-risk APEC and provide additional data on clonal type. It is clear from this and other studies that the APEC plasmid is highly prevalent in both broiler and turkey clinical *E. coli*

isolates, and previous work has demonstrated a clear role in fitness and virulence in birds and persistence within poultry barns. However, this alone may not identify high-risk APEC clones. Combining the presence of the APEC plasmid with clinically-dominating STs and the O78 serogroup, based on our results, provides more definitive proof that a strain can be classified as APEC with high virulence potential.

CONCLUSION

This work has resulted in the development of a revised APEC typing scheme, based on comprehensive data identifying dominant high-risk clones of interest. It will undoubtedly not identify all highly virulent APEC, other STs will likely arise in the future, and some strains within STs may become more virulent or successful than others. Thus, this scheme will likely need revisions as the landscape of APEC evolves. However, comprehensive analyses of the current APEC landscape indicate that this revised scheme will detect strains of highest risk to both turkey and broiler health.

REFERENCES

1. Johnson, T.J., Wannemuehler, Y., Doetkott, C., Johnson, S.J., Rosenberger, S.C., Nolan, L.K. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. *J Clin Microbiol* 46:3987-3996; 2008.

2. Wang, S., Meng, Q., Dai, J., Han, X., Han, Y., Ding, C., Liu, H., Yu, S. Development of an allele-specific PCR assay for simultaneous sero-typing of avian pathogenic *Escherichia coli* predominant O1, O2, O18 and O78 strains. *PLoS One* 9:e96904; 2014.

3. Johnson, T.J., Siek, K.E., Johnson, S.J., Nolan, L.K. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. *J Bacteriol* 188:745-758; 2006.

4. Mageiros, L., Meric, G., Bayliss, S.C., Pensar, J., Pascoe, B., Mourkas, E., Calland, J.K., Yahara, K., Murray, S., Wilkinson, T.S., Williams, L.K., Hitchings, M.D., Porter, J., Kemmett, K., Feil, E.J., Jolley, K.A., Williams, N.J., Corander, J., Sheppard, S.K. Genome evolution and the emergence of pathogenicity in avian *Escherichia coli*. *Nat Commun* 12:765; 2021.

Figure 1. Phylogenetic landscape of turkey clinical *E. coli*. The inner ring is colored by blinded companies participating in this study. The outer gray rings indicate dominant sequence types identified amongst clinical isolates.

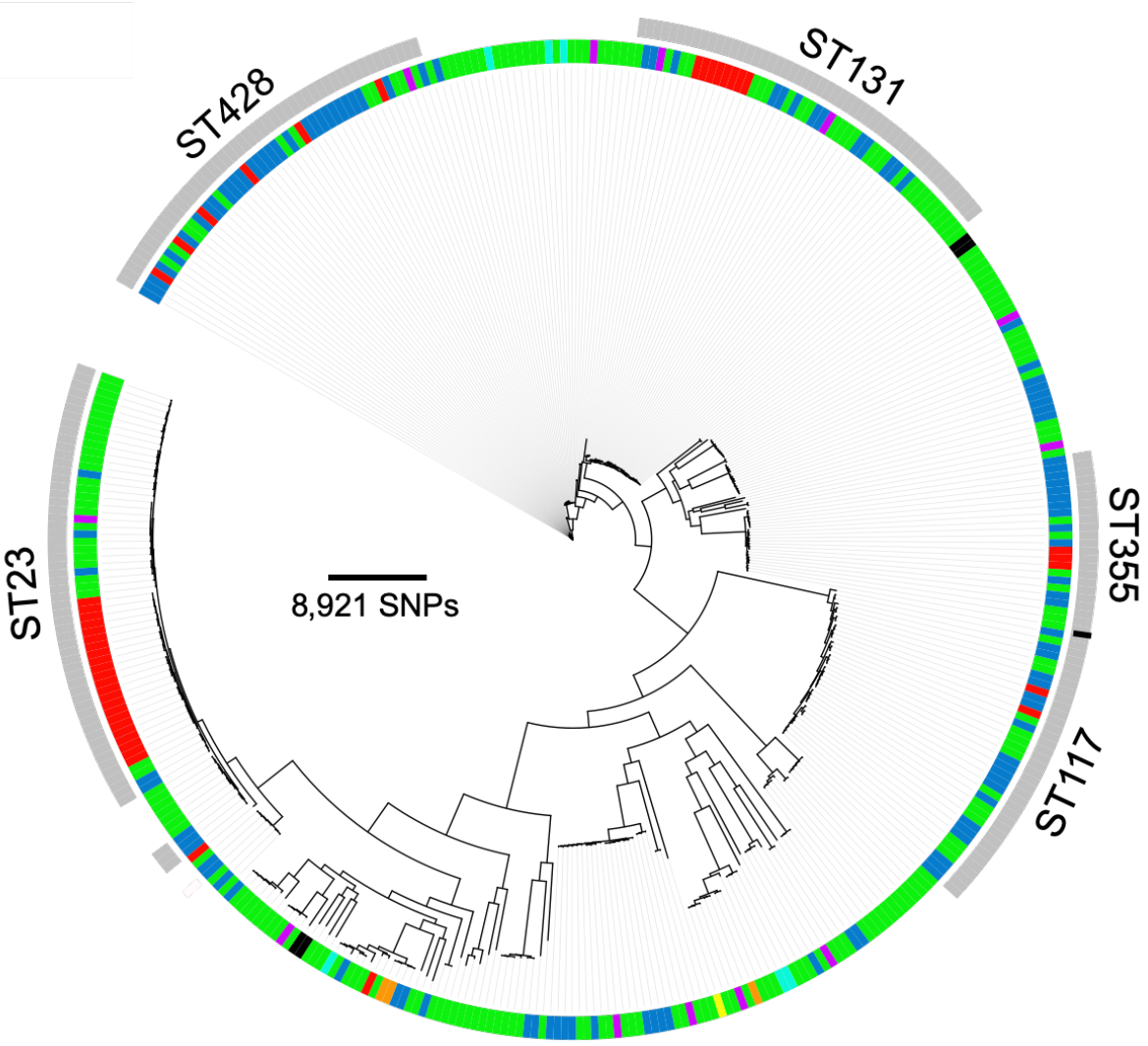
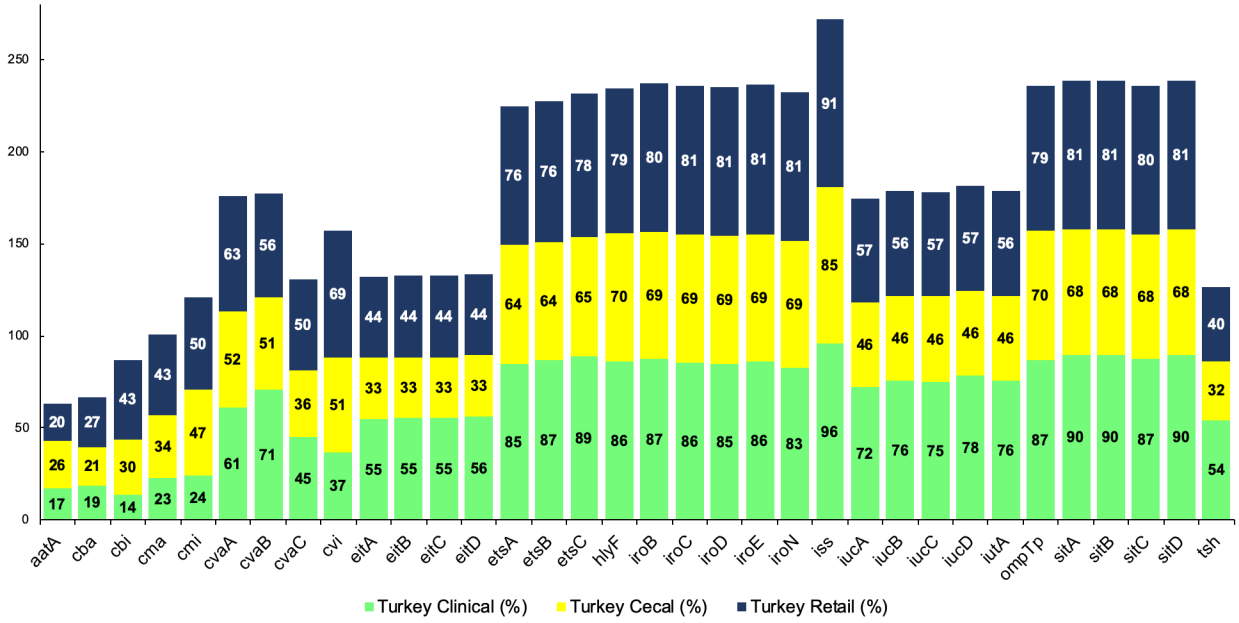


Figure 2. Patterns of prevalence of selected genes of the ColV plasmid PAI among turkey clinical (N=397), turkey cecal (N=562), and turkey retail (N=1,468) *E. coli*. Data is displayed using a stacked bar graph depicting population prevalence (%).



EXPLORING POSTBIOTIC-BASED STRATEGIES FOR THE MITIGATION OF AVIAN PATHOGENIC *ESCHERICHIA COLI* IN LAYERS

C. Logue[A], T. Johnson[BC], E. Gingerich[B], S. Riggs[B], and M. Farmer[B]

[A]University of Georgia, Department of Population Health, Athens, GA 30602
[B]Diamond V, Cedar Rapids, IA 52404
[C]University of Minnesota, Department of Veterinary and Biomedical Science, St Paul, MN 55108

SUMMARY

Avian pathogenic *Escherichia coli* (APEC) remains a significant bacterial burden to the layer industry worldwide. Transitions towards antibiotic-free and cage-free operations necessitate alternative control strategies against APEC. Here, a combination of controlled research and field observations were used to assess the ability of postbiotic technology to mitigate APEC challenge. A controlled challenge study was conducted where seven-day-old layer pullets were challenged orally or intratracheally with a marked virulent APEC strain. Gut colonization and lesion scores were assessed at seven days following challenge. Then, field flocks experiencing elevated mortality due to APEC were divided into a matched control versus postbiotic field trial, following mortalities from 16 to 40 weeks of age. Use of postbiotic in feed demonstrated significant reductions in APEC load and colibacillosis lesion scores compared to control. Postbiotic-fed birds also had reduced mortality counts compared to control. These results suggest that postbiotics may mitigate APEC challenges in commercial layers.

MATERIALS AND METHODS

Controlled trial. Specific Pathogen Free (SPF) layer chicks (White Leghorns) were commercially sourced (Charles River) and hatched at the PDRC hatchery facility. On day of hatch, 15 chicks were randomly assigned to each of eight isolator units (n = 120). Feed was supplied to the units as follows: four units were assigned a feed labelled as "control" and the remaining four units assigned a feed labelled as "test" (Table 1). All chicks had access to feed and water *ad libitum* with lighting on a 12 hour day/ night cycle. Temperature in the isolators was recorded daily and followed the normal schedule for chicks i.e., 95°F for the first seven days followed by reduction to 90°F in the second week and 85°F by week three with 80°F by week four.

All birds were weighed during the course of the study with weights of individual birds in each isolator recorded at day 0, 14, and 21. Weights were used to determine the mean and standard deviation at each sampling date for statistical analysis.

For challenge, a bacterial suspension of the challenge strain APEC O78 was prepared as follows. Glycerol frozen stock was removed and struck to tryptone soy agar (TSA) (Difco, Franklin Lakes, NJ) with incubation at 37°C for 18-24 h. A single colony was selected and inoculated into 5 mL of brain heart infusion (BHI, Difco) broth with incubation at 37°C for 18 h in a shaking incubator at 200 rpm (New Brunswick, Edison, NJ). The following day, 1 mL of the overnight culture was added to 50 mL of BHI and incubated at 37°C for approximately 3 h with shaking at 200 rpm. The OD_{600} was measured and recorded as 0.52-0.53. The remaining suspension was centrifuged at 6000 x g for 10 minutes to precipitate the cells and the supernatant discarded. The final suspension was re-suspended in 5 mL of sterile phosphate buffered saline (PBS, MP Biomedical, Irvine, CA) and placed on ice until use. Suspensions were made no more than 1 h before use. To confirm the inoculum dose, a 1.0 mL aliquot of the suspension was removed, serially diluted in PBS and plated on MacConkey agar (MAC, Difco) MAC and MAC supplemented with nalidixic acid (NAL 30 ug/mL) (Fisher Scientific, Pittsburg, PA). Plates were allowed to dry and incubated at 37°C for 18-24 h. Colony counts confirmed that the challenge dose was 1.2-1.4 x 10^8 cfu per 100 uL.

At 14 days of age, birds in each unit were challenged either orally or intratracheally with the challenge organism. Briefly, for oral challenge sterile gavage needles were attached to a sterile 1 mL syringe and the syringe loaded with the challenge strain. Using approved methods, the mouth was gently opened and the gavage needle placed down the back of the throat to deliver a challenge volume of 100 uL. Time to deliver and handling of the bird took no more than 30 seconds resulting in minimal distress for the bird. For intratracheal challenge, a similar approach was used but a sterile flexible 25G catheter (Exel International,

Redondo Beach, CA) was used and placed into the trachea to allow accurate delivery of 0.1 mL of the suspension into the upper airway. Good delivery was considered when there was a cough response from the bird. Time to deliver the challenge strain to the bird was less than 30 seconds and birds did not show any adverse signs of distress due to the inoculation protocol. All birds were returned to their respective isolators and monitored twice daily for signs of illness or distress consistent with infection and in such a case the birds were euthanized early per the IACUC approved protocol.

At seven days post challenge (21 day of age), all birds were euthanized using the AVMA approved method of CO_2 asphyxiation. Birds were aseptically opened and swabs of the air sacs and heart blood were obtained and streaked directly to MAC and MAC+NAL for isolation. In addition, samples of the liver, lung, spleen, and ceca were aseptically collected and placed in sterile Whirlpak sample collection bags (Whirl-Pak®, Nasco, Madison, WI). All samples were stored on ice until microbial analysis was carried out. All microbial analysis was carried out within 3 h of collection. All birds were scored for lesions on opening using the scoring system. Organs scored for lesions included thoracic air sacs, lungs, heart and pericardium, liver and spleen (see table 2). Lesions in necropsied birds were compared to pictures of typical scores to ensure accuracy of the scorer. All tissue samples collected were weighed and diluted 1:1 with sterile PBS; in the case of the spleen, the tissue was diluted 1:2 with PBS due to size of the sample. All samples were manually homogenized in the bag to ensure adequate mixing. 100 uL aliquots of the tissue suspensions were serially diluted in 0.9 mL volumes of PBS and 10 uL volumes for each dilution were plated out on MAC and MAC+NAL and allowed to dry. All samples were plated in triplicate for all dilutions analyzed. Plates were incubated at 37°C for 18-24 h and counted to determine Log_{10} cfu/g of tissue analyzed. Counts obtained were recorded and validated by confirmation of at least one colony per sample using two PCR reactions targeting the O78 for the challenge strain and 16S rRNA gene for *E. coli*. From the plate count isolates, a single colony of typical *E. coli* morphology was picked and struck to TSA with incubation at 37°C for 18-24 h.

All data was analyzed using Graph Pad with ANOVA used to compare difference across the treatment groups. All significance was accepted at $P< 0.05$. Statistical analysis was carried out for lesion scores, and plate counts from various organs as well as weight gain analysis.

Field trial. A layer company experiencing historically elevated mortalities due to *E. coli* was recruited to assess the impact of a continuously fed postbiotic application on reducing mortalities. To assess this, a matched control-treatment trial was conducted for five different flock cycles. Weekly mortalities were recorded for control versus treatment flocks. Feed conversion ratios, eggs per hen housed, and case weights were also recorded.

RESULTS

Controlled trial. In the APEC challenge groups, intratracheal APEC challenge induced significantly higher lesion scores in the control feed compared to PBS challenged birds ($P < 0.05$). Oral challenge numerically, but not statistically, increased lesion scores in the control feed groups. In the postbiotic-fed groups (test feed), lesion scores were significantly reduced compared to the control-fed groups under intratracheal challenge ($P < 0.05$). Lung lesion scores were numerically reduced for postbiotic-fed birds challenged intratracheally with APEC, compared to those receiving control feed. Live lesion scores were significantly reduced for postbiotic-fed birds challenged intratracheally with APEC, compared to those receiving control feed ($P < 0.05$). Similarly, heart lesion scores were significantly reduced for postbiotic-fed birds challenged intratracheally with APEC, compared to those receiving control feed ($P < 0.05$).

With oral challenge, postbiotic-fed birds had numerically reduced counts of the challenge strain compared to control-fed birds. With intratracheal challenge, postbiotic-fed birds had significantly reduced counts of the challenge strain compared to control-fed birds ($P < 0.05$). Total *E. coli* counts were reduced in orally challenged birds for postbiotic versus control groups; however, postbiotic treatment had no effects on total *E. coli* counts in intratracheally challenged birds. Reductions in the challenge strain counts were also observed in the postbiotic treatment groups compared to control groups in internal organs and the air sacs.

Field trial. The field trial utilized a layer operation that had exhibited elevated *E. coli* mortalities during a specific production window between approximately 25 and 35 weeks of age. Application of the postbiotic continuously in feed significantly reduced mortalities within this window ($P < 0.05$) across multiple flocks tested. Positive effects of the postbiotic were also observed with respect to feed conversion ratios and/or egg production.

CONCLUSION

This study demonstrates that the application of a postbiotic continuously in feed can exert positive

effects on the mitigation of *E. coli*, both with respect to overall *E. coli* load and the development of *E. coli* disease.

Figure 1. Phylogenetic landscape of turkey clinical *E. coli*. The inner ring is colored by blinded companies participating in this study. The outer gray rings indicate dominant sequence types identified amongst clinical isolates.

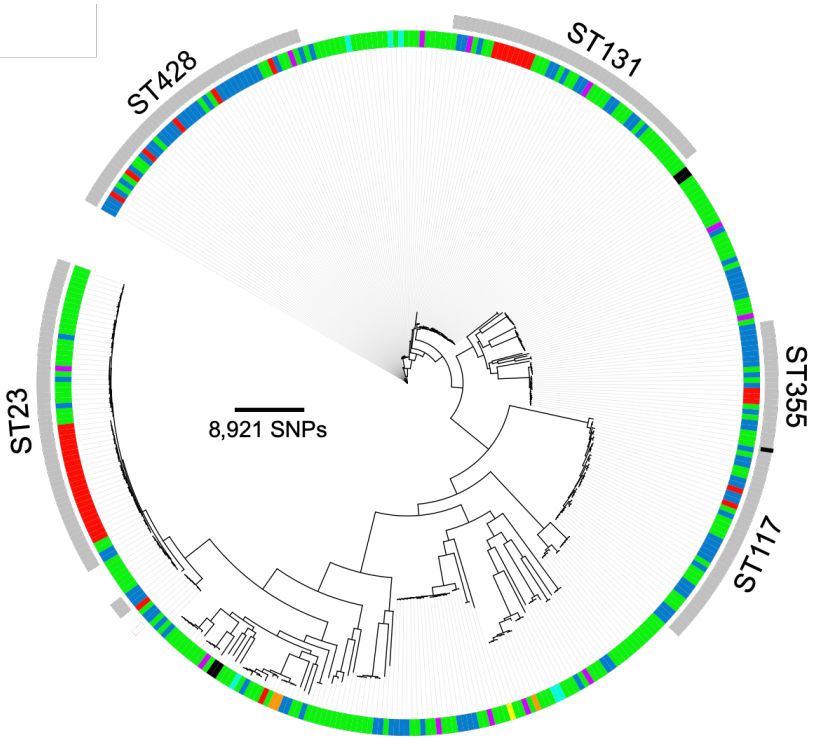
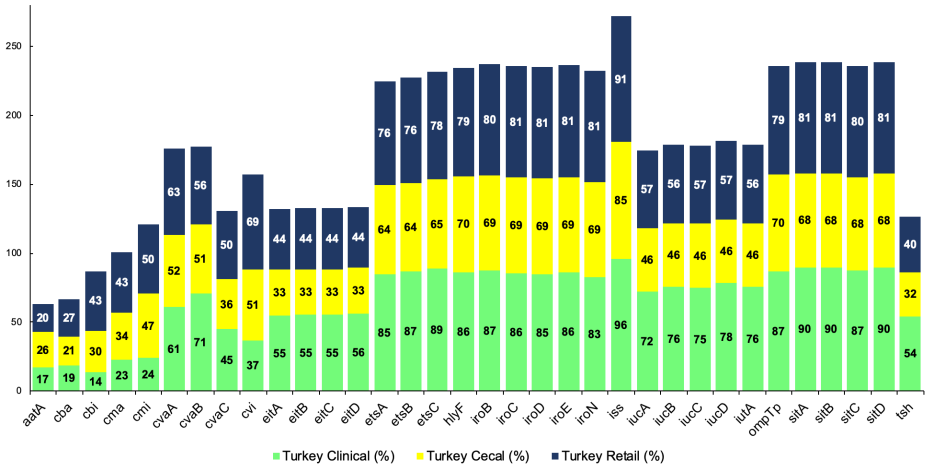


Figure 2. Patterns of prevalence of selected genes of the ColV plasmid PAI among turkey clinical (N=397), turkey cecal (N=562), and turkey retail (N=1,468) *E. coli*. Data is displayed using a stacked bar graph depicting population prevalence (%).



INVESTIGATING INFECTIOUS BRONCHITIS DMV/1639 VIRUS IN BROILER BREEDER ROOSTERS

K. Jones[A], R. Gilbert[B], M. Alfonso[A], P. Stayer[B], F. Hoerr[C], and R. Gallardo[D]

[A]Ceva Animal Health, Lenexa, KS
[B]Sanderson Farms, Inc. Laurel, MS
[C]Veterinary Diagnostic Pathology, LLC, Fort Valley, VA
[D]Dept. of Population Health & Reproduction, School of Veterinary Medicine, University of California Davis, Davis, CA

SUMMARY

DMV/1639 is a strain of infectious bronchitis virus (IBV) with a tropism for the respiratory (1), enteric (2-4), urinary (5-7), and reproductive tracts (8-12). Early oviduct infection with DMV1639 IBV has been associated with false layer syndrome (FLS), which is characterized by cystic or atretic oviducts, and a poor peak production in affected hen flocks (8, 9, 13, 14). This virus' effects in males, however, have not been previously reported. This is a case report of decreased reproductive performance due to male infertility associated with gross and microscopic testicular lesions in broiler breeder roosters. Roosters with urate pasting around the vent area were selected from houses with poor reproductive performance. Upon necropsy examination, they had grossly abnormal testes, mainly unilateral atrophy leading to marked testicular asymmetry. IBV antigen was detected in the testicular tissues via immunohistochemistry. Specifically, IBV strain DMV/1639 was detected via qRT-PCR in tissue pools (testes, kidneys, cecal tonsils, tracheas). In addition, this diagnosis was further supported by positive HI results indicating DMV/1639 as a likely culprit. This report suggests the role of IBV in roosters with impaired fertility concerns. Lastly, molecular surveillance of IBV to monitor emerging variants that can potentially affect the urogenital tracts of chickens is an important monitoring tool that should be utilized when troubleshooting fertility issues in breeder flocks.

INTRODUCTION

In the summer of 2021, two flocks of 37 and 38w old Ross 708 hens and Yield Plus males in central Texas were visited. The flocks had complaints of decreased egg production, declining egg fertility, broody hens, and males with urate-stained vents. On the 37w farm, there were four houses on the farm, but one was most affected. The affected house had an egg production of around 71% compared to the other three houses on the farm which were laying around 77% (breed standard for this age). Mortality was in the single digits in all houses for both males and females. There were no complaints of noticeably wrinkled eggs at either farm. This area of Texas has had concerns with multiple variant strains of IBV recently, including DMV/1639 and PA/1220/98- like strains, so IBV was a potential concern in these flocks. Both flocks had been vaccinated with IBV vaccines which included a live attenuated GA08 vaccine at day of age followed by a modified live Mass/Ark vaccine at 2 weeks, a live attenuated Mass/Conn/Ark vaccine at 6 weeks, a live attenuated Mass/Holland vaccine at 11 weeks, and two inactivated 4-way vaccines administered simultaneously at 15 weeks of age.

MATERIALS AND METHODS

During the visits, affected broody hens and urate vent-stained males were collected for necropsy examination. Birds were bled prior to euthanizing, and serum was separated after allowing for clotting time. During necropsy examination, fresh tissues (trachea, reproductive tracts (if present), kidney, cecal tonsils, and testes) were collected for qRT-PCR for IBV, and virology efforts, and testes were collected for histological examination and immunohistochemistry. Fresh tissues were shipped via overnight delivery with dry ice to the SSIU Ceva Laboratory in Lenexa, KS. Fixed tissues were sent to UC Davis at Davis, CA and Veterinary Diagnostic Pathology, LLC in Fort Valley, VA for processing and interpretation. Serum was submitted for testosterone levels (males only) to UC Davis at Davis, CA. Sera from a similar age unaffected flock was also collected for testosterone level comparison purposes. Post exposure sera was collected from the 38w flock visited when the affected flock was around 55w of age, and were submitted for IBV HIs to the PDRC at UGA in Athens, GA.

RESULTS

Necropsy. Necropsy examination of affected hens included reproductive tracts that appeared

juvenile in appearance as if they had never been in production. One hen that did have a reproductive tract had a small cystic structure attached to it. Necropsy of affected males revealed heavy urate staining around the abdomen and cloaca with moderate inflammation. These males also had a marked difference in their testicle size and/or appearance. Testicular atrophy, hyperemia, and congestion (often unilaterally) were the most prominent lesions noted.

IBV viral load. Cecal tonsils, kidneys, tracheas, oviducts, and testes from 37 and 38-week-old birds from the same flocks as the males showing testicular atrophy were tested for IBV by RT-qPCR. RNA extraction from organs was performed using the RNeasy Mini Kit (QIAgen, Valencia, CA). Multiple organs of the same type were pooled for testing. A screening RT-qPCR assay targeting the 5'UTR genomic region of IBV was initially performed (15). For IBV genotyping, a panel of RT-qPCR assays targeting the S gene was used to specifically detect genotypes DMV/1639 (16), Mass, Conn, Ark, DE072/GA98 (17), GA08, and GA13 (18). The virus quantification is presented as the cycle threshold (CT) value. CT values were compared using one-way ANOVA followed by Tukey's multiple comparisons test. Statistical differences were considered when $P<0.05$

The screening PCR targeting the 5'UTR shows that the highest viral load was detected in cecal tonsils. Birds from the 37-week-old flock showed overall higher viral loads than the 38-week-old birds. DMV/1639 was the most prevalent genotype among those tested in all sampled flocks, with 37-week-old birds being the most affected. Mass was detected in cecal tonsils from both age groups and kidneys from 38-week-old birds. The cecal tonsils and kidneys from the 38-week-old birds also tested positive for Ark. Conn, DE072/GA98, GA08 and GA13 strains were not detected in any of the flocks tested.

IBV HI's. HI titers were highest for DMV/1639 with titers ranging from 256-1024 and a GMT of 512, followed by Mass with titers ranging from 128-1024 and a GMT of 332. Other GMTs included 215 for Ark 99, 108 for Conn, and 10 for Del/072.

Histopathology and IBV IHC of testicles and testosterone quantification. These results are discussed separately in the paper presented by Dr. Rodrigo Gallardo in the virology section of this meeting. All help to support the suspicion that IBV is involved in the reproductive issues observed in the case flocks.

DISCUSSION

DMV/1639 is a strain of infectious bronchitis virus (IBV) with a tropism for the respiratory, urinary, and reproductive tracts. Early oviduct infection with DMV1639 IBV has been associated with false layer syndrome (FLS), which is characterized by cystic or atretic oviducts, and a poor peak production in affected hen flocks. This virus' effects in males, however, have not been previously reported. This report of cases involving decreased reproductive performance due to male infertility associated with gross and microscopic testicular lesions in broiler breeder roosters suggests the role of IBV, likely with strain DMV/1639. This report also demonstrates the importance of molecular surveillance of IBV to monitor emerging variants that can potentially affect the urogenital tracts of chickens as an important monitoring tool that should be utilized when troubleshooting fertility issues in breeder flocks.

(The full length article has been accepted for publication in *Avian Diseases*.)

REFERENCES

1. Jackwood MW, de Wit S. Infectious bronchitis. In: Swayne DE. Diseases of poultry. 14th ed. Hoboken (NJ): Wiley-Blackwell. p. 167-188; 2020.

2. Lucio B, Fabricant J. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. Avian Dis. 34(4):865-870; 1990.

3. Hauck R, Gallardo RA, Woolcock PR, Shivaprasad HL. A coronavirus associated with runting stunting syndrome in broiler chickens. Avian Dis. 60(2):528-534; 2016.

4. da Silva AP, Hauck R, Nociti SRC, Kern C, Shivaprasad HL, Zhou H, Gallardo RA. Molecular biology, and pathological process of an infectious bronchitis virus with enteric tropism in commercial broilers. Viruses. 13(8):1477; 2021.

5. Meulemans G, van den Berg TP. Nephropathogenic avian infectious bronchitis viruses. World Poult Sci J. 54(2):145-153; 1998.

6. Benyeda Z, Mató T, Süveges T, Szabó E, Kardi V, Abonyi-Tóth Z, Rusvai M, Palya V. Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. Avian Pathol. 38(6):449-456; 2009.

7. Gelb J, Jr., Ladman BS, Pope CR, Ruano JM, Brannick EM, Bautista DA, Coughlin CM, Preskenis LA. Characterization of nephropathogenic infectious bronchitis virus DMV/1639/11 recovered from Delmarva broiler chickens in 2011. Avian Dis. 57(1):65-70; 2013.

8. Broadfoot DI, Pomeroy BS, Smith WM. Effects of infectious bronchitis in baby chicks. Poult Sci. 35(4):757-762; 1956.

9. Crinion RA, Hofstad MS. Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis. 16(2):351-363; 1972.

10. de Wit JJ, Nieuwenhuisen-van Wilgen J, Hoogkamer A, van de Sande H, Zuidam GJ, Fabri TH. Induction of cystic oviducts and protection against early challenge with infectious bronchitis virus serotype D388 (genotype QX) by maternally derived antibodies and by early vaccination. Avian Pathol. 40(5):463-471; 2011.

11. Parent E, Gagnon-Francoeur A, Lanthier B, Hébert G, Buczinski S, Boulianne M. Diagnostic accuracy of ultrasonography to detect false layers in a commercial laying flock infected by an infectious bronchitis virus Delmarva genotype causing cystic oviducts. Avian Dis. 64(2):149-156; 2020.

12. da Silva AP, Giroux C, Sellers HS, Mendoza-Reilley A, Stoute S, Gallardo RA. Characterization of an infectious bronchitis virus isolated from commercial layers suffering from false layer syndrome. Avian Dis. 65(3):373-380; 2021.

13. Jones RC, Jordan FT. The exposure of day-old chicks to infectious bronchitis and the subsequent development of the oviduct. Vet Rec. 87(17):504-505; 1970.

14. Duff RH, Macdonald JW, McMartin DA, Ross JG. Infection of day-old chicks with infectious bronchitis (IB) virus and subsequent anatomical abnormalities. Vet Rec. 88(12):315; 1971.

15. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, Jackwood MW. Development and evaluation of a real-time TaqMan RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J Virol Methods. 138(1-2):60-65; 2006.

16. Gelb Jr J, Jackwood MW. Infectious bronchitis. In: Williams SM, Dufour-Zavala LDES, Jackwood MW, Lee MD, Lupiani B, Reed WM, Spackman E, Woolcock PR. A laboratory manual for the isolation, identification, and characterization of avian pathogens. 6th ed. Athens (GA): American Association of Avian Pathologists (AAAP). 2016.

17. Roh HJ, Hilt DA, Jackwood MW. Detection of infectious bronchitis virus with the use of real-time quantitative reverse transcriptase-PCR and correlation with virus detection in embryonated eggs. Avian Dis. 58(3):398-403; 2014.

18. Mo J, Angelichio M, Gow L, Leathers V, Jackwood MW. Validation of specific quantitative real-time RT-PCR assay panel for infectious bronchitis using synthetic DNA standards and clinical specimens. J Virol Methods. 276:113773; 2020.

GENOMIC COMPARISON OF DMV-LIKE AND QX-LIKE INFECTIOUS BRONCHITIS VIRUS STRAINS: IS THERE ANY RELATIONSHIP BETWEEN GENE SEQUENCES AND TISSUE TROPISM?

R. Jude[A], A. da Silva[A], R. Uttarwar[A], R. Beckstead[B], and R. Gallardo[A]

[A]Poultry Medicine laboratory, School of Veterinary Medicine, University of California, Davis
[B]Ceva Animal Health

SUMMARY

To determine if specific genes other than the spike (S) gene of the IBV viral genome are related to pathogenicity or tissue tropism, whole genome sequences from four DMV-like and five QX-like isolates were obtained by Illumina sequencing. The nine sequences from our study were compared to other IBV sequences on GenBank using the basic local alignment search tool (BLAST). Each IBV gene (ORF1a, ORF1ab, S, 3a, 3b, envelope (E), membrane (M), 5a, 5b, and nucleocapsid (N)) was analyzed individually. Nucleotide and amino acid identities were compared, as well as insertions and deletions in each gene. Phylogenetic analyses were performed for the evolutionary relationships between strains.

INTRODUCTION

Infectious bronchitis is an upper respiratory disease of chickens caused by the coronavirus infectious bronchitis virus (IBV). The viral genome is nearly 27 kbp long and comprises 10 genes that encode for over 20 viral proteins. The spike (S) gene is currently used for IBV classification (1), but differences in other parts of the viral genome might play a role in pathogenicity or tissue tropism. IBV primarily affects the chicken upper respiratory tract, but some strains, such as DMV/1639 and QX, can cause urogenital disorders (2-5). We hypothesize that these non-respiratory clinical presentations of certain IBV strains may be explained by additional variations among their genes and not solely by the relationship of their spike gene.

MATERIALS AND METHODS

Isolates, Library Preparation, and Sequencing. Allantoic fluid from amplification of four DMV-like isolates (CTE-5, DMV25, -26, and -28) with urogenital tropism were collected from the United States and 5 QX-like isolates (HU, GR, SR, FR, CN), again with urogenital tropism, were collected from Hungary, Greece, Israel, France, and China (2). Viral RNA was extracted from the allantoic fluid of each isolate using TRIzol LS Reagent (Invitrogen) and Direct-zol RNA MiniPrep Plus (Zymo Research). Whole genome cDNA libraries were prepared from the isolated viral RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and prepared libraries were sequenced by Illumina HiSeq.

S1 classification. The S1 subunit of the IBV S gene was extracted from the 9 whole genome sequences obtained and phylogenetic classification according to Valastro (1) was performed. Briefly, the S1 subunit sequence from 161 whole genome sequences were obtained from the NCBI database and, in addition to the 9 S1 sequences used in this study, were aligned using the MAFFT v7.450 plugin (6) for Geneious Prime 2022.0.2 (Biomatters Ltd.). A phylogenetic tree was built using the RAxML 8.2.11 plugin (7) in Geneious Prime using the GTR GAMMA I nucleotide model and 1000 bootstrap replicates.

Gene sequence comparisons. Whole genome sequences from the nine isolates and from 129 GenBank BLAST sequences that were QX-like (n=96) or DMV-like (n=33) were separated by open reading frames (ORFs) to obtain sequences for each of the 10 IBV genes (ORF1a, ORF1ab, S, 3a, 3b, envelope (E), membrane (M), 5a, 5b, and nucleocapsid (N)) using Geneious Prime. Each gene sequence was BLAST searched and the top 5 hits based on nucleotide percent identity were identified. Genes for each isolate were sorted with BLAST for hits greater than or less than 95% identity. For each gene, two separate alignments using the MAFFT v7.450 plugin were made for the DMV-like isolates and GenBank sequences or for the QX-like isolates and GenBank sequences. For all 10 genes, insertions and deletions in the nine isolates were identified based on the consensus for each alignment. From each alignment, the RAxML 8.2.11 plugin was used to build phylogenetic trees using the GTR GAMMA I nucleotide model and 1000 bootstrap replicates.

RESULTS

S1 classification. All 5 QX-like isolates belong to genotype 1 (GI) lineage 19, while the 4 DMV-like isolates belong to GI lineage 17 using the current S1 classification system.

Gene nucleotide insertion and deletion analysis. No insertions or deletions were detected in genes 3a, 5a, 5b, or N from any of the nine isolates. For gene 1ab, there were no insertions or deletions in any isolate except the QX-like FR which had a 3 nt insertion and 3 nt deletion. The number of insertions and deletions for each additional gene of the nine isolates may be seen in Table 1.

Gene nucleotide identities. For the 4 DMV-like isolates, CTE 5-1 had <95% identities for the 1a and spike genes while DMV25, -26, and -27 had less than 95% identity for the envelope gene. Meanwhile, for the QX-like isolates, CN and FR had no top BLAST hits of less than 95%. Only the 1a gene of GR had top hits with <95% identity. For HU and SR, genes 5a, 5b, membrane, and spike were >95% identity as well as the envelope gene for SR. All other genes for HU and SR had top hits of <95%. Additionally, DMV-like strains shared genes which were more closely related to turkey coronaviruses from the U.S. and Canada while QX-like strains shared genes with other fowl coronaviruses from hosts such as guineafowl, teal and partridges across Eurasia.

Gene phylogenies. Using spike gene, DMV25, -26, -28, and CTE 5-1 were closely related to other DMV strains and, likewise, the QX-like isolates from this study were also all closely related to other QX strains (Figure 1). For all other gene phylogenies, DMV25, -26, and -28 were generally divergent from CTE-5 while the 5 QX-like isolates largely showed high diversity from each other, aside from envelope gene (Figure 2).

DISCUSSION

Our investigation of DMV-like and QX-like IBV isolates, which have shown to present with urogenital and renal tropism, indicated that while both strain types may be classified distinctly using the Valastro S1 subtype system, there is diversity amongst the isolates for other genes despite them belonging to the same lineages. For example, CTE 5-1, which belongs to the same lineage as DMV25, -26, and -28, was a unique DMV-like strain with its closest relative only sharing 91% identity in the S1 gene. In addition, the diversity seen between other genes may be explained by different vaccination protocols in Eurasia and North America which could be responsible for shaping evolutionary patterns. It has been seen that many conserved genes are preserved between vaccine and wild-type strains, even though the S gene might be completely different. Based on nucleotide identities for the DMV-like and QX-like strains, aside from spike gene, the nucleocapsid and envelope genes were the most divergent and warrant additional investigation.

In our analysis, there was great diversity between the QX-like isolates compared to the DMV-like isolates. The explanation for this diversity may be multifaceted. First, DMV25, -26, and -28 isolates used in this study were highly similar and therefore resulted in a smaller number of BLAST hits to compare. Meanwhile, as QX-like strains are largely found in Europe and Asia, researchers in these locations have submitted significantly more sequences to NCBI than North American scholars where DMV-like strains may be found; thus, there is an inherent database bias. Finally, DMV-like strains are newly emerging compared to QX-like strains which have been circulating longer and therefore have been able to generate more variants.

Additionally, regarding the incidence of DMV-like isolates sharing high gene similarities with turkey coronaviruses in N. America and QX-like isolates sharing those with other fowl coronaviruses across Eurasia, this diversity among hosts could again be related to the fact that GenBank has a disproportionate number of QX-like strains compared to DMV-like strains. However, there may also be more commingling of wild birds with commercial poultry in Europe and Asia that could explain these differences.

(The full-length article will be published in *Avian Diseases*.)

REFERENCES

1. Valastro V, Holmes EC, Britton P, Fusaro A, Jackwood MW, Cattoli G, Monne I. S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. Infect Genet Evol. 39:349-364; eng. 2016 Apr.

2. Benyeda Z, Mató T, Süveges T, Szabó E, Kardi V, Abonyi-Tóth Z, Rusvai M, Palya V. Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. Avian Pathol. 38:449-56; eng. 2009 Dec.

3. Gelb J, Jr., Ladman BS, Pope CR, Ruano JM, Brannick EM, Bautista DA, Coughlin CM, Preskenis LA. Characterization of nephropathogenic infectious bronchitis virus DMV/1639/11 recovered from Delmarva broiler chickens in 2011. Avian diseases. 57:65-70; 2013.

4. Broadfoot DI, Pomeroy BS, Smith WM. Effects of Infectious Bronchitis in Baby Chicks. Poultry Science. 35:757-762; 1956.

5. de Wit JJ, Nieuwenhuisen-van Wilgen J, Hoogkamer A, van de Sande H, Zuidam GJ, Fabri TH. Induction of cystic oviducts and protection against early challenge with infectious bronchitis virus serotype D388 (genotype QX) by maternally derived antibodies and by early vaccination. Avian Pathol. 40:463-71; eng. 2011 Oct.

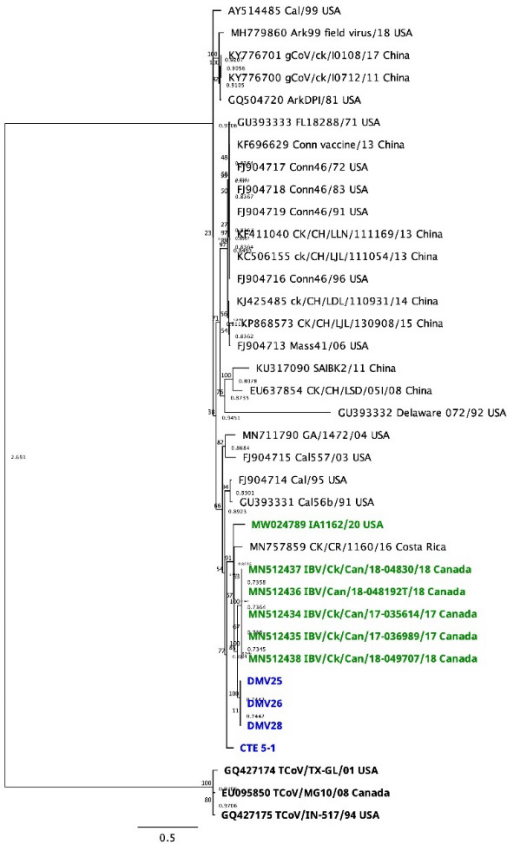
6. Katoh K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Molecular Biology and Evolution. 30:772-780; 2013.

7. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 30:1312-1313; 2014.

Table 1. Nucleotide insertions/deletions in select IBV genes.

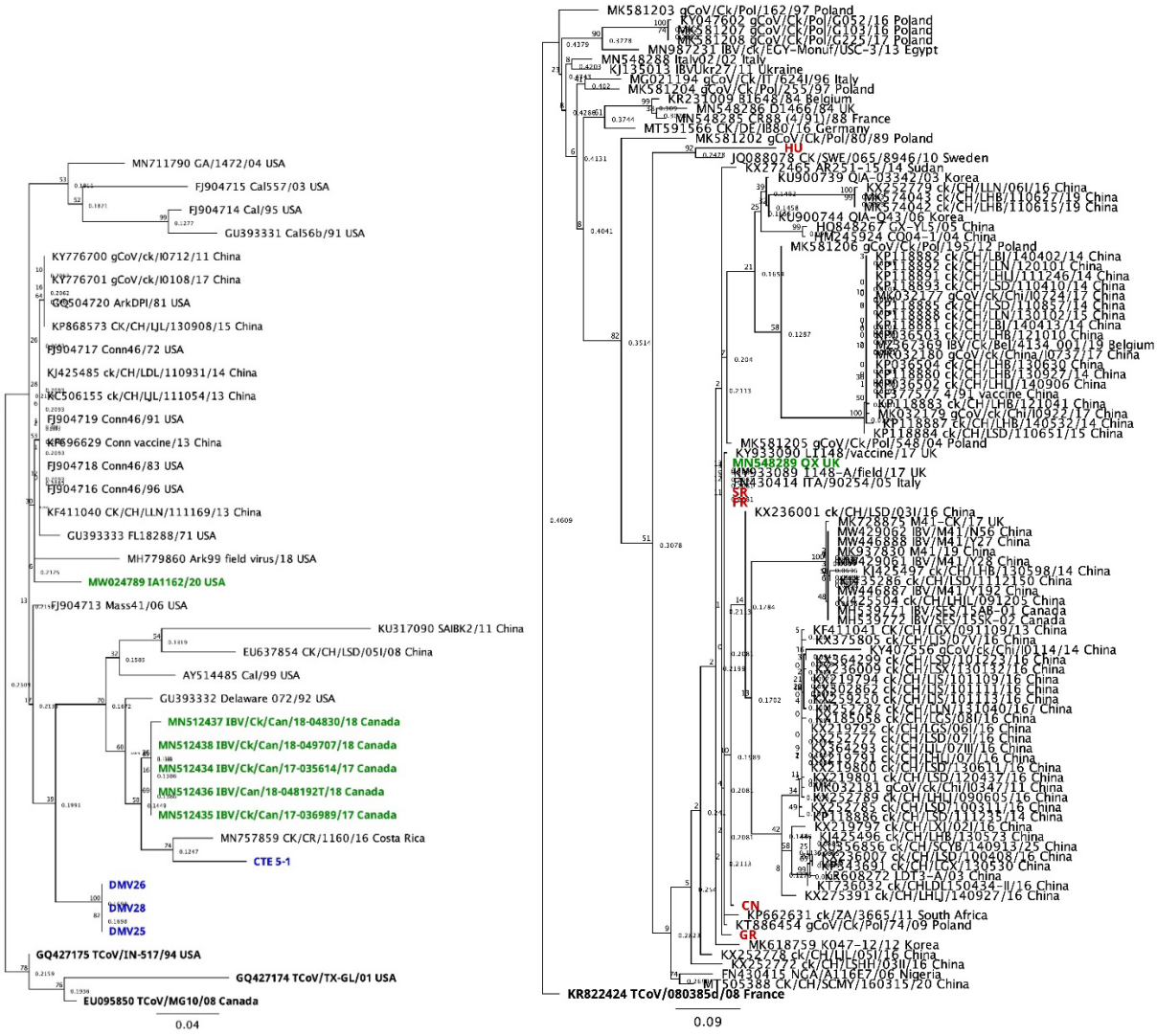
		1a	S	3b	E	M
QX-like	CN	9/12	12/3	3/-	9/-	9/-
	FR	9/18	12/3	3/-	9/-	9/-
	GR	3/33	12/3	3/-	9/-	9/-
	HU	3/9	12/3	-/-	9/-	9/-
	SR	3/12	12/3	3/-	9/-	9/-
DMV-like	CTE 5-1	3/-	24/12	-/-	6/-	6/-
	DMV 25	-/9	24/12	-/-	-/-	-/-
	DMV 26	-/9	24/12	-/-	-/-	-/-
	DMV 28	-/9	24/12	-/-	-/-	-/-

Figure 1. DMV-like (left) and QX-like (right) phylogenetic trees using the spike gene.




MT591566 CK/DE/IB80/16 Germany
MN548286 D1466/84 UK
MK574042 ck/CH/LHB/110615/19 China
MK574043 ck/CH/LHB/110627/19 China
KR231009 B1648/84 Belgium
MN548288 Italy02/02 Italy
FN430415 NGA/A116E7/06 Nigeria
CN
MN548289 QX UK
FN430414 ITA/90254/05 Italy
KP662631 ck/ZA/3665/11 South Africa
KX272465 AR251-15/14 Sudan
KU900739 QIA-03342/03 Korea
MK618759 K047-12/12 Korea
KR822424 TCoV/080385d/08 France
0.5

Figure 2. DMV-like (left) and QX-like (right) phylogenetic trees using the envelope gene.



CLOSTRIDIUM PERFRINGENS AND *EIMERIA MAXIMA* CO-INFECTION EFFECTS SECRETORY CELL DIFFERENTIATION IN BROILER CHICKENS

S. Kinstler[A], J. Maurer[A], E. Wong[A], C. Hofacre[B], and M. Lee[C]

[A]Virginia Tech, Department of Animal and Poultry Sciences, Blacksburg, VA 24060,
[B]Southern Poultry Research Group, Athens, GA 30607
[C]Biomedical Science and Pathobiology, Blacksburg, VA 24060

SUMMARY

Necrotic enteritis (NE) is a significant intestinal disease in the broiler chicken industry that affects producer economics and animal welfare annually. NE is caused by *Clostridium perfringens* but is predisposed by coccidiosis when infected with the parasite *Eimeria* causing various severities of infection. These pathogens cause intestinal damage that results in reduced feed intake, weight loss, and mortality. Our central hypothesis is *C. perfringens* and *Eimeria* co-infection damages the intestinal epithelium, preventing nutrient absorption by rupturing enterocytes and birds respond to intestinal atrophy through upregulation of stem cell proliferation and differentiation to rebuild intestinal villi and restore function. Using *in-situ* hybridization and qPCR, we examined intestinal morphology and gene expression of signaling pathways that induce stem cell differentiation and proliferation after infection of *C. perfringens, Eimeria,* or a co-infection with both *Eimeria* and *C. perfringens*. Intestinal morphology was altered with co-infection of *Eimeria* and *C. perfringens*, increasing crypt depth and shortening villi to reduce digestion and absorption capabilities, resulting in lower body weights of these birds. Gene expression of stem cell markers did not change in the presence of infection, indicating that the observed increase in crypt depth could be a delay of stem cell differentiation. Damage to intestinal villi, causing a blunting effect that decreases the amount of enterocytes available for nutrient absorption, could contribute to reduced gene expression of secretory cells. A better understanding of the effect of infection with *Eimeria* and *C. perfringens* on intestine function and morphology could contribute to development of targeted treatment methods.

INTRODUCTION

NE is an intestinal bacterial disease that significantly impacts modern poultry production, resulting in economic losses over an estimated $6 billion annually (1). NE is caused by the spore-forming, obligate anaerobe, *Clostridium perfringens* that is ubiquitous in the poultry house environment and chicken gastrointestinal tract and under homeostatic circumstances, is regulated by the microbiome (2). For NE to occur, birds are initially infected with the protozoan *Eimeria* that is specific to different segments of the intestine, invades intestinal epithelial cells, and cycles through asexual and sexual reproduction to develop oocysts shed in the feces for ingestion by other birds. *Eimeria maxima* is often parasitized in the jejunum, the mid-small intestine responsible for most digestion and nutrient absorption (3). *Eimeria* ruptures intestinal enterocytes during reproduction, inhibiting nutrient absorption, and producing gross lesions associated with NE. In severe cases of NE, a sudden spike in morbidity indicates the presence of infection related to clinical NE, permitting management to intervene and treat any remaining birds (4). Subclinical NE infections are less severe and display subtle clinical signs, if any, but still cause disruption of the intestine structure and functions (5). Rupturing of enterocytes by *Eimeria* paired with production of *C. perfringens* toxins is believed to avert intestinal repair and prolong illness (3). Additionally, the inhibition of nutrient absorption caused by enterocyte damage results in poor feed conversion, lengthens production time, and increases feed costs. To control NE, a better understanding of the molecular and cellular events that occur in the intestine during NE is needed to regulate this disease.

The intestinal epithelium composition includes specialized cells that harbor receptors involved in nutrient absorption and transport (6). Developmental pathways are crucial for maintaining these cells through cell turnover under homeostatic conditions and replace the entire intestinal epithelium every three to four days. Olfactomedin-4 (*Olfm4*) is a glycoprotein expressed in the small intestine that acts as a stem cell marker (6). In the intestine, stem cells are localized to the base of the crypts and differentiate into functional cell types as they move up the villus. Differentiation of absorptive or secretory cells depend

on cell fate determination through activation of cell signaling pathways including Notch and Wnt/β-catenin (7). These pathways are paramount to cell regeneration and repair through stimulation of stem cell proliferation and differentiation. Notch signaling induces formation of absorptive enterocytes whereas Wnt signaling stimulates secretory cell production, including mucus-secreting Goblet cells. Various intestinal diseases influence proliferation of stem cells through manipulation of Wnt and Notch signaling (8). These pathways are emerging as important targets of gram-positive bacterial infections but are not well understood in NE infections.

Cases of subclinical enteritis diminish digestion and absorption of nutrients in a subtle manner and can go unnoticed until birds are taken for processing. Additional feed costs for birds to get to market weight, lower yields, and rejection of birds during processing lead to devastating economic losses for producers. In addition, consumers face food safety concerns if infected birds are not rejected, or processing equipment is contaminated. To alleviate economic losses and prevent food-related illnesses, a better understanding of subclinical NE infections and mechanisms of the pathogens involved can contribute to prevention or treatment of this disease.

MATERIALS AND METHODS

To determine the effects of NE pathogens on intestine morphology and function, a NE challenge model was used. On d0, 144 male chicks were allotted into 12 Petersime battery cages using a randomized complete block design. Chicks were initially sexed at the hatchery and placed in a solid-sided barn maintained under ambient humidity. Each cage was assigned to one of three blocks and four treatments were included within each block. In each cage, 12 birds were placed to total 36 birds per treatment. Feed and water were available *ad libitum* throughout the trial with each cage containing one trough feeder and one trough drinker. Chicks were fed a non-medicated commercial-type broiler starter diet compounded according to NRC guidelines.

Cages within blocks were assigned to four treatments; 1. Non-challenged control, 2. *Eimeria* infection, 3. *Clostridium perfringens* infection, 4. *Eimeria* and *C. perfringens* co-infection. Birds infected with *Eimeria* or co-infected with *Eimeria* and *C. perfringens* were gavaged with approximately 1,000 sporulated oocysts of *E. maxima* per bird on d14. Birds infected with *C. perfringens* or co-infected with *Eimeria* and *C. perfringens* received a 1.0 mL gavage of *C. perfringens* with 1 x 10^8 cfu/bird on day 19 to achieve peak infection on day 21. Target NE for co-infection of *Eimeria* and *C. perfringens* was less than 10%, representing a subclinical infection. During *Eimeria* or *C. perfringens* inoculation, birds not receiving an infection that day were gavaged with 1.0 mL of distilled water.

On day 21, two birds from each cage were weighed and necropsied. Body weights were averaged within cage and gross lesions were scored on a scale of 0 to 3 (Lesion score 0 = normal, Lesion score 1 = slight mucus covering small intestine, Lesion score 2 = necrotic small intestine mucosa, Lesion score 3 = Sloughed and blood small intestine mucosa and contents). Whole tissue sections of jejunum were collected and fixed in formalin for histology. After fixation, whole tissues were mounted in paraffin blocks and cut onto microscope slides for *in-situ* hybridization. Scrapings of jejunum mucosa were collected from remaining jejunum for qPCR analysis.

To examine intestinal morphology, *in-situ* hybridization using the RNAscope 2.5 HD Assay-Brown kits by Advanced Cell Diagnostics were used on jejunum section of 4 birds per treatment to localize gene expression. A probe designed for *Olfm4* staining was used to stain 3-5 sections from each bird. Sections were imaged to measure intestine morphology characteristics including villus height, crypt depth, and villus height:crypt depth (VH:CD). Crypt depth was measured in µm using *Olfm4* staining to localize stem cells from the base of the crypt to the end of *Olfm4* staining along the villi. Villus height was measured in µm from the end of the crypt to the tip of the villi and VH:CD was determined based on these measurements. VH:CD and crypt depth measurements were normally distributed and statistical analysis used Tukey HSD in JMP Pro 16. Villus height was non-normally distributed and non-parametric analysis was implemented using Steel-Dwass All Pairs test.

Gene expression was analyzed using qPCR after RNA extraction of jejunum mucosa and cDNA synthesis using 6 birds per treatment. RNA was extracted using Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) and normalized to 200 ng/µL before synthesis of cDNA using Peltier Thermal Cycler (Bio Rad, Hercules, CA). A 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA) was used to analyze genes of interest. *Olfm4* and *Lgr5* were used as stem cell markers and *NFKB1, NOX1,* and *MyD88* were used as inflammation markers. *Atoh1* and *Muc2*, a goblet cell marker, were analyzed to determine secretory cell differentiation and *Notch1*, *Hes1,* and *Pept1* to represent the absorptive cell lineage. Each sample was run in duplicate and *RPLP0* and *RPLP4* were used as reference genes. Data was normalized using lognormal transformation and statistical analysis was completed using Tukey HSD.

RESULTS

Gene expression of stem cell markers *Olfm4* and *Lgr5* did not change with infection of *C. perfringens, Eimeria*, or co-infection ($P > 0.05$) as shown in Table 1. Absorptive cell differentiation was not affected by infection, but secretory cell differentiation was downregulated in *Atoh1* and *Muc2* ($P < 0.01$). Infection of *C. perfringens* increased *Atoh1* and *Muc2* expression, but co-infection of *Eimeria* and *C. perfringens* not different than the non-challenged control group. Infection of *Eimeria* did not change expression of *Atoh1* expression compared to the *C. perfringens* infection and the non-challenged control, but expression was greater than birds co-infected with *Eimeria* and *C. perfringens*. Expression of *Muc2* was greater after infection of *C. perfringens* ($P < 0.01$), but no differences were detected between the *Eimeria* infection, co-infection with *Eimeria* and *C. perfringens,* and the non-challenged control. Inflammation markers *NFKB1, NOX1,* and *MyD88* did not change expression between the non-challenged control and introduction of infections or co-infection ($P > 0.05$).

Intestinal morphology in birds co-infected with *E. maxima* and *C. perfringens* was severely altered, increasing crypt depth, shortening villi, and decreasing in VH:CD ($P < 0.001$). *Eimeria* infection, *C. perfringens* infection, and *Eimeria* and *C. perfringens* co-infection blunted villus height compared to the non-challenged control as shown in Table 2. Villi were shortest in birds co-infected with Eimeria and *C. perfringens*. Villus height of birds infected with *C. perfringens* was similar to co-infection villus height but was similar to birds infected with *Eimeria*. Crypt depth increased in birds infected with *Eimeria* or co-infected with *Eimeria* and *C. perfringens* compared to the non-challenged control and *C. perfringens* infection. The extension of crypt depth paired with shortening of villus height during infection resulted in changes to VH:CD. *Eimeria* and *C. perfringens* co-infection resulted in significantly decreased VH:CD because crypt depth increased, and villus height was shortened ($P < 0.001$). VH:CD decreased in *Eimeria* and *C. perfringens* infections compared to the non-challenged control, but VH:CD in the *Eimeria* infection was reduced compared to the *C. perfringens* infection.

Body weight decreased with *Eimeria* and *C. perfringens* co-infection ($P = 0.01$) and was not different among *Eimeria* infection, *C. perfringens* infection, and the non-challenged control. Lesion scores ranged from 0 to 1, indicating a subclinical infection was achieved with the absence of gross lesions correlating to severe NE. Gross lesions with a score of 1 were exclusively in birds co-infected with *Eimeria* and *C. perfringens*; however, no significant differences were seen among infections.

DISCUSSION

In this study, inoculation of NE pathogens to induce infection or co-infection did not affect stem cell proliferation as hypothesized but did impact secretory cell differentiation during co-infection. Increased expression of *Atoh1* in the jejunum of birds infected with *C. perfringens* indicated an increase in differentiation to the secretory cell lineage. An increase in expression of the goblet cell marker, *Muc2*, confirmed secretory cell differentiation and function. In birds co-infected with *Eimeria* and *C. perfringens*, downregulation of *Atoh1* expression implies a decrease in secretory cells development, inferring downregulation of *Muc2* expression. However, a significant decrease in *Muc2* expression compared to the non-challenged control was not detected. Notch signaling markers *Notch1* and *Hes1* did not change in expression during infection indicating that nutrient absorption was not affected by infection in the remaining enterocytes. Blunting of villi reduces the number of enterocytes present but did not decrease mRNA abundance of nutrient transporters in fewer enterocytes present compared to the non-challenged control after co-infection. This could imply an upregulation of nutrient transport in the remaining enterocytes and other nutrient transporters would be of interest to examine.

An increase in crypt depth in birds co-infected with *Eimeria* and *C. perfringens* was not due to a change in stem cell over proliferation and instead could be a delay of differentiation, resulting in visibly longer crypts. The shortening of villus height observed could be caused by an increase in sloughing of the intestinal epithelium caused by *Eimeria* rupturing enterocytes and *C. perfringens* toxins damaging the epithelium. Differences in morphology were drastic in this subclinical infection, proving that birds without the more severe clinical NE symptoms experience intestinal damage and reduced surface area for nutrient absorption, lowering body weight. Expression of nutrient transporters on enterocytes besides *Pept1* are of interest to determine if absorption of a particular nutrient is favored, or if overall absorption is inhibited. In the secretory lineage, differentiation of cells other than goblet cells, such as Paneth cells or enteroendocrine cells, could be decreased by the pathogens to prevent production of regulatory molecules secreted to induce the immune response by the host. Therefore, decreasing secretory cell differentiation could be a mechanism of pathogenesis in NE and a potential target for intervention.

REFERENCES

1. Wade B, Keyburn AL. The true cost of necrotic enteritis. *Poultry World* [accessed 2022 January 31]. https://www.poultryworld.net/Meat/Articles/2015/10/The-true-cost-of-necrotic-enteritis-2699819W/; 2015.

2. Immerseel FV, Buck JD, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. Clostridium perfringens in poultry: An emerging threat for animal and public health. *Avi Path*. 33(6), 537-549; 2004.

3. Williams RB. Intercurrent coccidiosis and necrotic enteritis of chickens: Rational, integrated disease management by maintenance of gut integrity. *Avi Path.* 34(3), 159-180; 2005.

4. M'Sadeq SA, Wu S, Swick RA, Choct M. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. *Ani Nutr*. 1(1), 1–11; 2015.

5. Skinner JT, Bauer S, Young V, Pauling G, Wilson J. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. *Avi Dis*. 54(4), 1237–1240; 2010.

6. Zhang H, Wong E. Identification of cells expressing Olfm4 and Lgr5 mRNA by in situ hybridization in the yolk sac and small intestine of embryonic and early post-hatch chicks. *Poult Sci.* 97(2), 628-633; 2018.

7. Takahashi T, Shiraishi A. Stem cell signaling pathways in the small intestine. *Int Jour Mol Sci.* 21(6); 2020.

8. Silva-García O, Valdez-Alarcón JJ, Baizabal-Aguirre VM. Wnt/β-catenin signaling as a molecular target by pathogenic bacteria. *Front Immun.* 10; 2019.

Table 1. Co-infection of *E. maxima* and *C. perfringens* decreases secretory cell gene expression without effecting stem cell proliferation or differentiation of absorptive cells.

[1]Each value represents the least-square means of the fold change mRNA abundance from 5-6 birds per treatment.

	Stem cells		**Enterocytes (absorptive)**			**Goblet cells (secretory)**	
Treatments	Olfm4	Lgr5	Notch1	Hes1	Pept1	Atoh1	Muc2
Non-challenged control	1.25	1.06	1.08	1.15	1.44	1.42^{bc}	1.06^{b}
***C. perfringens* infection**	1.20	1.59	1.71	0.93	1.67	2.97^{a}	2.21^{a}
***Eimeria* infection**	0.70	1.07	1.56	0.84	1.44	2.10^{ab}	1.24^{b}
Co-infection[3]	1.93	1.14	1.17	0.80	0.99	1.07^{c}	0.78^{b}
Standard Error	0.52	0.16	0.22	0.17	0.22	0.33	0.24
P - value	0.23	0.16	0.11	0.62	0.08	<0.01	<0.01

Day 21 Control treatment values were used as the calibrator

[2]Data was log transformed to conform to normality for statistical analysis

[3]Co-infection of *Eimeria* and *C. perfringens*

[a,b,c]Means in the same column with different superscripts are statistically different ($P < 0.05$) based on Tukey HSD mean separation

Table 2. Co-infection of *Eimeria* and *C. perfringens* increases crypt depth, shortens villus height, and decreases VH:CD in broiler chickens.

Treatments	Villus Height (µm)	Crypt Depth (µm)	VH:CD
Non-challenged control	1126.32[a]	120.77[b]	9.74[a]
***C. perfringens* infection**	873.60[bc]	122.14[b]	7.21[b]
***Eimeria* infection**	981.16[b]	189.98[a]	5.24[c]
Co-infection[2]	540.54[c]	194.31[a]	2.91[d]
Standard Error	44.15	8.51	0.47
P - value	<0.001	<0.001	<0.001

[1]Each value represents the least-square means of measurements taken from 3-5 sections cut from 3-4 birds per treatment.
[2]Co-infection of *E. maxima* and *C. perfringens*
[a,b,c,d]Means in the same column with different superscripts are statistically different ($P < 0.05$) based on Tukey HSD mean separation or Steel-Dwass All Pairs

HISTOMONIASIS OUTBREAKS IN COMMERCIAL TURKEYS IN BRITISH COLUMBIA, CANADA

G. Wing Lin

Canadian Poultry Consultants Ltd. Abbotsford, BC, Canada

SUMMARY

In 2020, there was a significant increase in the number of histomoniasis cases in commercial turkeys in British Columbia resulting in devastating economic impacts on the turkey industry. With the lack of approved efficacious treatment for histomoniasis, prevention has been strongly reliant on strategies including practicing stringent biosecurity, enhancing management practices, minimizing concurrent disease stressors based on an understanding of the epidemiology of histomoniasis. The present report describes the multi-farm histomoniasis outbreak in British Columbia and discuss some of the potential risk factors for histomoniasis outbreaks observed in some of the reported cases.

INTRODUCTION

Histomoniasis, commonly known as blackhead disease, is a protozoal disease reported in gallinaceous birds caused by the protozoan parasite *Histomonas meleagridis. H. meleagridis* has a complex interaction with the vectors in its existing environment. Although *H. meleagridis* is short-lived and fragile outside the host, its environmental stability can be prolonged by physical protective vectors such as common earthworms and cecal worms (*Hererakis* spp.) eggs. Particularly, when encapsulated by thick-shelled cecal worm eggs, the protozoan can survive within the eggs and remain infective for severe years. Incidence of histomoniasis has been reported in chickens, turkeys, peafowls, and other gallinaceous birds. When compared to chickens, the mortality and morbidity in turkeys are generally much more severe. Turkeys that are infected with *H. meleagridis* are often found depressed and anorexic. Sulfur-colored droppings may be found in birds with systemic infection. Characterized gross lesions of histomoniasis include enlarged, thickened ceca filled with caseous cores and/or necrotic debris. A "bullseye" lesion in the liver resulted from hepatic necrosis. Histopathology and/or PCR test are useful to confirm the diagnosis (1,2).

DESCRIPTION OF THE PROBLEM

In British Columbia, Canada, the number of histomoniasis cases reported in commercial turkey flocks has increased significantly over the last two years. In 2020 alone, there were 20 cases reported compared to six cases reported in 2019 (Figure 1). Over 95% of the cases were in the Fraser Valley Regional District, a poultry-dense geographical area. The unprecedented multi-farm outbreak of histomoniasis has serious economic and welfare consequences for the turkey industry with farm gate losses estimated at close to 1 million Canadian dollars in 2020 (British Columbia Turkey Marketing Board, pers. comm). Unfortunately, since the removal of Histostat-50® (nitarsone, 4-nitrophenyl-arsonic acid; Zoetis, Florham Park, NJ) in 2016 from the Canadian market, there has been a lack of approved efficacious treatment for histomoniasis (3,4). As a result, it is especially crucial to investigate the epidemiology of local histomoniasis outbreaks so that preventative strategies can be identified to minimize future outbreaks. To help identified potential risk factors for histomoniasis outbreaks, field visits were carried out at multiple histomoniasis positive turkey farms throughout 2020.

DISCUSSION

The Fraser Valley Regional District in British Columbia is one of the most poultry-dense geographical areas in Canada. Like many poultry farms in the area, most of the farms with recent histomoniasis outbreaks were located within one km of other poultry farms that housed long-live commercial poultry including table-egg layers, broiler breeders, and nonregulated small flocks. Chicken is known as the best host for cecal worm (*Heterakis gallinarum*) and thus a reservoir of *H. meleagridis* (2,5,6). As a result, the close inter-connected poultry industry may serve as vehicles for the physical transmission of the cecal worm eggs encapsulated histomonads from one farm to another. Three of the farms with recurrent histomoniasis outbreaks had both table-egg layers and meat turkeys on the same premise with barns less than 100 meters apart. In addition, the potential risk of carrying histomonads from one

facility to another increased with shared equipment and workers within the same premise. In cases where the proximity of poultry facilities cannot be avoided, it is especially important to have farm workers educated on practicing enhanced biosecurity. Routine deworming with an approved dewormer should be included as a regular protocol amongst all poultry commodities to help reduce overall cecal worm load in the area.

The sighting of a significant amount of vermin including earthworms and darkling beetles was another common finding on farms with histomoniasis. One of the farms with recurrent outbreaks of histomoniasis had a severe darkling beetle infestation. Colonies of live darkling beetles were found embedded along cracks and crevices throughout the walls and foundations. Darkling beetle can serve as a mechanical vector of cecal worm eggs along with histomonads (7). The common earthworm has been shown to consume and carry infective larval stage of the cecal worms, thus serving as a vector (1,2). To reduce earthworm access to the barn, some producers added physical partitions such as plastic tarps and plywood to block doorways. Chemical barriers including salt and lime were seen applied long perimeters to deter earthworms and insect vectors. Although the use of pesticides had shown some extent in reducing pest activity on some farms; the development of a custom-built integrated pest management plan should be considered to help address various challenges associated with pest control on individual farms.

Many of the field investigations were conducted over rainy seasons. Poor water drainage and water paddling along the barn perimeters were not uncommon on farms that had recent and recurrent histomoniasis outbreaks. Rainfall, topography, and soil consistency between farms can influence earthworm movement and water run-off, hence potentially bringing contaminated water, soil, and vermin from one site to another (5). Having gravel flooring along the outdoor perimeters improves water drainage and reduces water pooling when compared to soil and grass. Gravel floor also eliminates natural habitats for earthworms. Routine inspections and maintenance of the barn perimeters and foundations not only help to prolong structural longevity but also reduce the risk of contaminated moisture from entering the barn. To minimize water run-off and water pooling, new barn development should consider sealing the foundation properly and whenever possible, build the barn at a relatively higher elevation.

Many of the farms that had recurrent histomoniasis outbreaks showed challenges in practicing stringent biosecurity. Thoroughly cleaning and disinfection is especially difficult to achieve on farms that have multi-age flocks housed in inter-connected barns. The practice of all-in-all-out was not feasible unless the farm ceased production for an extended period. Prolonged manure storage was another common observation. Especially in cases where the stored manure was uncovered, wildlife and vermin can reintroduce contaminated manure to the subsequent flocks housed nearby. Based on discussions with the producers, the definitions of "thorough cleaning and disinfection" often vary widely. Up-to-date, continuous education on biosecurity practice can help to enhance the industry's overall biosecurity.

Like many other disease challenges, it was not surprised to learn that histomoniasis were more commonly reported in flocks that had concurrent disease challenge and/or on-farm environmental stressors. Multiple histomoniasis cases were diagnosed within two to three weeks after flocks were moved from one barn to another. Over the transition period, not only that the birds experienced a period of feed and water interruption, but they also had to acclimatize to their new environmental parameters. Other challenges reported include colibacillosis, enteritis, poor litter conditions, and heat-related stress.

CONCLUSIONS AND EXPANDED STUDIES

In November 2020, a multifaceted collaborative study was launched by the British Columbia Ministry of Agriculture, Food and Fisheries and the British Columbia Turkey Marketing Board to help understand the epidemiology of the local histomoniasis outbreaks, risk factors for the disease, and offer recommendations on methods for prevention. The projects are still in progress and results will be tentatively published in summer 2022. In the meantime, with the lack of approved efficacious treatment for histomoniasis, the applications of prevention strategies based on the understanding of the epidemiology of histomoniasis remain an important aspect of disease control.

REFERENCES

1. Hess, M., McDougald, L. R. Histomoniasis. In: Swayne, D. E. Diseases of Poultry, 14th Edition. Ames (IA): Wiley-Blackwell. p. 1223-1230; 2020.

2. McDougald, L. R. Blackhead disease (histomoniasis) in poultry: a critical review. Avian Dis, 49: 462-476; 2005.

3. Regmi, P. R., Shaw, A. L., Hungerford, L. L., Messenheimer, J. R., Tong Zhou, Pillai, P., Omer, A., Gilbert, J, M. Regulatory considerations for the approval of drugs against histomoniasis (blackhead disease) in turkeys, chickens, and game birds in the United States. Avian Dis, 60(4): 725-730; 2016.

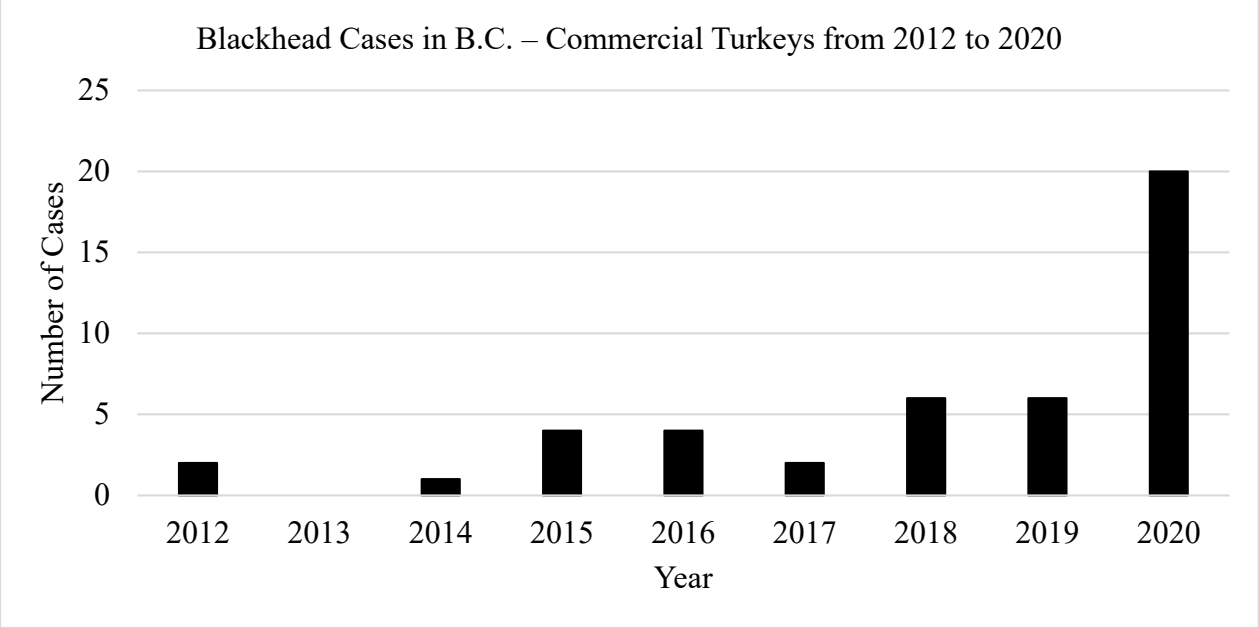
4. Liebhart, D., Ganas, P., Sulejmanovic, T., and Hess, M. Histomonosis in poultry: previous and current strategies for prevention and therapy. Avian Pathol, 46(1): 1-18; 2017.

5. Jones, R. E., Rivers, D. V., Fletcher, O. J., and Martin, M. P. Histomoniasis outbreaks in commercial turkeys in the southeastern United States: proximity of broiler breeder farms as a potential risk factor in disease development. J Appl Poult Res, 29(2): 496-501; 2020.

6. Kendall, S. B. The occurrence of Histomonas meleagridis in Heterakis gallinae. J Parasitol, 49: 169-172; 1959.

7. Beckmann, J. F., Dormitorio, T., Oladipupo, S. O., Terra, M. T. B., Lawrence, K., Macklin, K. S., and Hauck, R. Heterakis gallinarum and Histomonas meleagridis DNA persists in chicken houses years after depopulation. Vet Pathol, 298: 2021.

Figure 1. The number of confirmed histomoniasis cases in commercial turkeys between 2012 and 2020 (Personal communication, British Columbia Turkey Marketing Board).



PROTECTION INDUCED BY IBDV VACCINE STRAINS ST-12 AND 51A/C4 VERSUS A DELAWARE E CHALLENGE

A. Banda[A], R. Mackey[A], and F. Wilson[B]

[A]Mississippi State University, Poultry Research and Diagnostic Laboratory, Pearl, MS 39208
[B]Mississippi State University, Mississippi Veterinary Research and Diagnostic Laboratory, Pearl, MS 39208

SUMMARY

To evaluate the efficacy of an infectious bursal disease virus (IBDV) live vaccine containing the ST-12 and 51A/C4 strains preceding a challenge with a Delaware E strain, one hundred and five one-day old specific pathogen free birds were assigned into three experimental groups with the following treatments: a) non-vaccinated/non-challenged, b) vaccinated/challenged, and c) non-vaccinated/challenged. At day of hatching, the birds were vaccinated by coarse spray using a commercial vaccination cabinet and then distributed into experimental groups inside Horsfall-Bauer isolators. At 28 days of age, birds were challenged with a Delaware E strain. Eleven days after challenge, necropsy procedures were carried out. Only six birds (38.88%) of the vaccinated and challenged group showed gross bursal atrophy in comparison with 36 birds (100%) of the nonvaccinated and challenged group. Significant statistical differences were observed between the bursal/body weight ratios of the non-vaccinated/non-challenged (5.00), the vaccinated/challenged (3.75) and the non-vaccinated/challenged (1.19) groups. An average lymphoid depletion score of 3.8, between moderate to marked was observed in the non-vaccinated/challenged group, with significant difference ($P<0.001$) in comparison with the non-vaccinated/non-challenged and the vaccinated/challenged group. According to these results, the strains ST-12 and 51A/C4 provided protection vs the Delaware E challenge.

INTRODUCTION

Infectious bursal disease (IBD) is a viral disease widely disseminated in poultry that induces dysfunction of the immune system. The virus replicates in the bursa and induces bursal atrophy, compromising the mechanisms involved in antibody production. Chickens suffering from this disease are immunosuppressed, are more susceptible to opportunistic infections, and may develop a poor response to vaccinations. The most effective way to control IBD is by vaccination. There are different types of vaccines, with different characteristics and levels of attenuation. Live attenuated vaccines replicate in bursal tissue, and some low attenuated vaccine strains can induce destruction of the lymphoid tissue of the bursa, with development or atrophy. Live attenuated vaccines can be applied *in-ovo*, by drinking water, or by coarse spray (1, 2, 3).

Coarse spray vaccination is frequently used in the hatchery plants to immunize young birds against different viral diseases. The main advantage for this method is that a high number of birds can be immunized in a short time without being individually handled; therefore, the level of stress is reduced significantly.

The purpose of this study was to evaluate the efficacy of a vaccine that contains a blend of two intermediate strains of bursal disease virus (ST-12 and 51A/C4) applied by coarse spray in the face of a challenge with a Delaware E IBDV strain according to 9CFR Part 113 Section 113.331 (4).

MATERIALS AND METHODS

Study location. This experiment was conducted in the Vivarium at Mississippi State University's Poultry Research and Diagnostic Laboratory. This is a two-level restricted-access area operating under negative pressure and HEPA filtration.

SPF Eggs and incubation conditions. Specific pathogen free (SPF) fertile eggs were obtained from Charles River Laboratories (Wilmington, MA). Eggs were incubated under standard conditions. Only healthy and strong chickens were included in the trials; weak, small, or abnormal chickens were excluded. One hundred and eight birds were assigned into three groups with six replicates of the following treatments:

1. Non-vaccinated and non-challenged
2. Vaccinated and challenged
3. Non-vaccinated and challenged

IBDV maternal antibodies determined by ELISA. At day of hatching, blood samples from 29 chickens were collected and antibody titers vs IBDV were determined by BioCheck's ELISA IBDV Antibody Test Kit.

Vaccine and vaccination procedures. A vaccine containing a blend of two intermediate strains

of bursal disease virus, ST-12 (grown in tissue culture) and 51A/C4 (grown in chicken embryos), was evaluated in this study. At day of hatching, SPF birds were vaccinated by coarse spray using a commercial cabinet (Spraycox X®)

Challenge virus and procedures. An infectious bursal disease virus (IBDV) variant Delaware E strain was used for challenge purposes. At 28 days of age, birds were challenged with 2.2 $\log_{10}$ EID_{50} by eye drop in both eyes (0.03 mL per eye).

Data collection. Eleven days after challenge, birds were euthanized by CO_2 inhalation, and necropsy procedures were carried out. Body and bursal weights were recorded, and bursal/body weight ratios were calculated to characterize bursal atrophy (5). Bursal tissues were collected for histopathological evaluation.

Histopathological evaluation of bursal tissues. Bursal tissues fixed in 10% buffered formalin were paraffin embedded and sections were stained with standard hematoxylin and eosin staining. Parameters used in the evaluation of microscopic lesions associated with IBDV included: lymphoid depletion, lymphocytic/histiocytic infiltration, necrosis, scalloping, and fibrosis. Bursal sections were given a subjective severity score using a range of 0 to 5 as follows: 0 = Absent; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked, and 5 = Severe.

Statistical analysis. Body weight, bursal weight, bursal to body weight ratios, and bursal indexes were compared using a single factor analysis of variance followed by Tukey's test. When data did not meet normality and variance homogeneity, nonparametric methods were utilized.

Histopathological lesion scores were compared by Kruskall-Wallis test, followed by comparisons among groups by Dunn's test. Significance was determined at the $P<0.05$ level.

Animal use and biosafety policies. All animal procedures involved in this study were approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC),

RESULTS

Maternal antibody titers. Twenty-nine serum samples obtained at the day of hatching tested negative by ELISA.

Body weight. Average body weights were 491.58 g (non-vaccinated/non challenged group), 474.72 g (vaccinated/challenged group) and 457.77 g (non-vaccinated/challenged group) (Table 1). There was a significant difference between the non-vaccinated/non-challenged group and the non-vaccinated/challenged group ($P<0.01$).

Bursal weight. Group comparison of average body weights are included in Table 1. The average weights for non-vaccinated/non-challenged, vaccinated/challenged and non-vaccinated/challenged groups were 2.47 g, 1.78 g, and 0.545 g, respectively. There were significant statistical differences among all three groups.

Bursal/body weight ratio. Comparison of average bursal/body weight ratios are included in Table 1. There were significant statistical differences between the average bursal/body weight ratios of the non-vaccinated/non-challenged (5.00), the vaccinated/challenged (3.75) and the non-vaccinated/challenged (1.19) groups.

Birds with bursal atrophy. Six birds of the vaccinated and challenged group and 36 birds of the nonvaccinated and challenged group exhibited severe bursal atrophy. No bursal atrophy was observed in the non-vaccinated/non challenged control. Significant statistical differences were observed among all groups.

Lymphoid depletion. Lymphoid depletion histological scores are shown in Table 2. An average lymphoid depletion score of 3.8, between moderate to marked, was observed in the non-vaccinated/challenged group, with significant differences($P<0.001$) when compared with the non-vaccinated/non-challenged and the vaccinated/challenged groups.

Necrosis/Apoptosis. Necrosis and apoptosis scores are exhibited in Table 2. Average scores of necrosis and apoptosis between mild to moderate were observed in the three experimental groups. There was a significant difference only between the non-inoculated/non-challenged group (average score of 2.52) and the non-inoculated/challenged group (average score of 2.13) ($P<0.01$).

Mononuclear infiltrate. Average scores of mononuclear infiltrates including lymphocytes and macrophages are included in Table 2. Significant differences were observed in all the group comparisons. Average mononuclear infiltrate was between moderate and marked (average score of 3.19) in the non-vaccinated/challenged group. The non-vaccinated/non-challenged and vaccinated/challenged groups exhibited average scores of 1.27 and 1.93, respectively, between minimal and moderate.

Scalloping. Scalloping average scores are presented in Table 2. Significant differences were observed in all the group comparisons. The non-vaccinated/challenged group had an average score of 3.17 (between moderate to severe).

Other histopathological parameters such as lymphoid follicular hyperplasia, granulomatous foci, granulomas, and micro abscess were also evaluated, but were not analyzed since the values were minimal.

DISCUSSION

The purpose of this study was to evaluate the efficacy of a vaccine with two intermediate strains of bursal disease virus (ST-12 and 51A/C4) in SPF birds versus a challenge with a Delaware E strain.

Gross bursal atrophy was observed in six birds of the vaccinated/challenged group and in 100% of the birds in the non-vaccinated and challenged group. Furthermore, there were statistical differences in the bursal weight, and bursal/body ratio between the three groups. Moderate to marked average scores of lymphoid depletion, mononuclear infiltrate, and scalloping were observed in the non-vaccinated and challenged group, whereas minimal to moderate scores were observed in the vaccinated and challenged group. This indicates that even though six vaccinated birds developed bursal atrophy, the protective immunity induced by this vaccine was significant as compared to 100% of birds with bursal atrophy of the non-vaccinated and challenged group. The vaccine also elicited significant protection against lymphoid depletion, mononuclear infiltrate, and scalloping, when compared with the non-vaccinated and challenged group. Finally, there was a negative impact of the challenge on body weight, and this negative impact was lower in the vaccinated birds.

The test was valid because 100% of the placebo vaccinated, challenge control birds developed remarkable gross bursal atrophy due to IBDV at time of necropsy. According to the results obtained in this study, the vaccine provided satisfactory protection against IBDV. However, the SPF birds used in this study were highly susceptible to IBDV infection, given that they had no maternally-derived antibodies vs IBDV.

REFERENCES

1. Alkie TN, Rautenschlein S (2016) Infectious bursal disease virus in poultry: current status and future prospects. Vet Med (Auckl) 7:9-18. doi:10.2147/vmrr.S68905

2. Cheville NF (1967) Studies on the pathogenesis of Gumboro disease in the bursa of Fabricius, spleen, and thymus of the chicken. Am J Pathol 51 (4):527-551

3. Swayne DE (2020) Diseases of poultry. Fourteenth edition. ed. Wiley-Blackwell, Hoboken, NJ

4. 9 CFR 113.331 - Bursal Disease Vaccine. (2012) https://www.govinfo.gov/app/details/CFR-2012-title9-vol1/CFR-2012-title9-vol1-sec113-331. Accessed 08-05-2021

5. Lucio B, Hitchner SB (1979) Infectious Bursal Disease Emulsified Vaccine: Effect upon Neutralizing-Antibody Levels in the Dam and Subsequent Protection of the Progeny. Avian Diseases 23:466

Table 1. Average values of bursal weight, bursal weight, and bursa/body weight ratio.

	Non-vaccinated and non-challenged group	Vaccinated and challenged group	Non-vaccinated and challenged group
Body Weight	491.80±46.39[a]	474.72±45.46[ab]	457.77±38.73[b]
Bursal Weight	2.47±0.69[a]	1.78±0.76[b]	0.54±0.09[c]
Bursa/Body Weight Ratio	5.00±1.18[a]	3.75±1.50[b]	1.19±0.22[c]

Table 2. Average Scores[A] of histological lesions: Lymphoid Depletion, Necrosis/Apoptosis, Mononuclear Infiltrate, and Scalloping.

	Non-vaccinated and non-challenged group	Vaccinated and challenged group	Non-vaccinated and challenged group
Lymphoid Depletion	2.18±0.36[a]	2.53±0.73[a]	3.80±0.34[b]
Necrosis/Apoptosis	2.52±0.52[a]	2.33±0.37[ab]	2.13±0.49[b]
Mononuclear infiltrate	1.28±0.54[a]	1.93±0.53[b]	3.19±0.37[c]
Scalloping	1.80±0.45[a]	2.31±0.81[b]	3.17±0.44[c]

[A] Score system: 0 = Absent; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked, and 5 = Severe

IMPACT OF LACTIC ACID BACTERIA ON EGGSHELL QUALITY OF MOLTED COMMERCIAL LAYERS

V. Marcano[A] and A. Welsher[A]

[A]Elanco Animal Health, 2500 Innovation Way, Greenfield, IN 46140 USA

SUMMARY

Probiotics are live microbial supplements that confer health benefits to poultry by improving intestinal homeostasis (1-4). Probiotics act by competitive exclusion, immune modulation, strengthening of the intestinal barrier, promoting villi length, and producing molecules such as neurochemicals, enzymes and organic acids, ultimately improving nutrient absorption (5). Commonly probiotics include bifidobacterial and/or lactic acid producing bacteria (6-8). Lactic acid bacteria (LAB) are gram positive, non-spore producing bacterial strains with probiotic potential (9, 10). LAB act by producing lactic acid in the intestine, which reduces the intestinal pH, becoming an energy source for the bird and aiding in microflora balance and gut integrity (5, 11). LAB such as *Pediococcus acidilactici* are an important tool to maintain performance during periods of high stress. *P. acidilactici* supplementation in laying hens has been found to improve feed conversion ratio, egg weight, and eggshell thickness (12-15). In this study, we evaluated the use of an in-water *Pediococcus* spp. probiotic in commercial laying hens undergoing molting.

MATERIALS AND METHODS

Pediococcus spp. was applied via drinking water using a dosing pump, delivered to Hy-Line W-36 hens for nine consecutive days every eight to 10 weeks beginning at 53 weeks of age until 98 weeks of age. The hens were housed in a conventional A-frame caged facility. Production parameters evaluated included livability, case weight, as well as shell quality. Bird health was evaluated via necropsies of five to six birds two weeks after each application. The performance of the *Pediococcus* spp. treated group ("T" group) was compared to a flock of the same breed previously housed at the farm (non-molted, "N" group), as well as to another molted flock from the same company ("C" group) of a different breed (Lohmann LSL).

RESULTS AND CONCLUSIONS

Laying hens from the T group had improved feed conversion compared to birds from the N group. Feed conversion visibly improved after application of *Pediococcus* spp. via the drinking water. Overall, the T group had better body weight uniformity compared to the N group. Laying hens from the T group had sustained body weight uniformity and livability compared to laying hens from the C group, especially after molting. The change in percent egg production after molt was not as drastic in laying hens from the T group, compared to laying hens from the C group. Necropsy showed that laying hens from the T group had a sustained number of ova (>5 per hen) until at least 91 weeks of age. Overall, grade outs remained above 80% of the life for the flock. Results show that *Pediococcus* spp. via the drinking water can be a valuable tool to minimize stress during molting of laying hens.

REFERENCES

1. R. Fuller, Probiotics in man and animals. *J Appl Bacteriol* 66, 365-378 (1989).

2. R. Havenaar, Huis in't Veld JHJ (1992) Probiotics: a general view. *Wood BJB, ed*, 209-224 (1992).

3. F. a. A. O. (FAO), *Probiotics in food : health and nutritional properties and guidelines for evaluation*. FAO food and nutrition paper, 85 (World Health Organization, Rome, 2006).

4. F. Guarner, G. Schaafsma, Short communication Probiotics. Int. *Jour of Food Microbiol* 39, 237-238 (1998).

5. M. E. Abd El-Hack *et al.*, Probiotics in poultry feed: A comprehensive review. *J Anim Physiol Anim Nutr (Berl)* 104, 1835-1850 (2020).

6. G. R. Gibson, M. B. Roberfroid, Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of nutrition* 125, 1401-1412 (1995).

7. S. Seifert *et al.*, Modulation of innate and adaptive immunity by the probiotic Bifidobacterium longum PCB133 in turkeys. *Poult Sci* 90, 2275-2280 (2011).

8. K. C. Mountzouris *et al.*, Evaluation of the efficacy of a probiotic containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult Sci* 86, 309-317 (2007).

9. W. H. Holzapfel, P. Haberer, J. Snel, U. Schillinger, J. H. H. in't Veld, Overview of gut flora and probiotics. *International journal of food microbiology* 41, 85-101 (1998).

10. G. Klein, A. Pack, C. Bonaparte, G. Reuter, Taxonomy and physiology of probiotic lactic acid bacteria. *International journal of food microbiology* 41, 103-125 (1998).

11. J. Tarradas, N. Tous, E. Esteve-Garcia, A. J. Brufau, The Control of Intestinal Inflammation: A Major Objective in the Research of Probiotic Strains as Alternatives to Antibiotic Growth Promoters in Poultry. *Microorganisms* 8, (2020).

12. D. Mikulski, J. Jankowski, M. Mikulska, V. Demey, Effects of dietary probiotic (Pediococcus acidilactici) supplementation on productive performance, egg quality, and body composition in laying hens fed diets varying in energy density. *Poult Sci* 99, 2275-2285 (2020).

13. D. Mikulski, J. Jankowski, J. Naczmanski, M. Mikulska, V. Demey, Effects of dietary probiotic (Pediococcus acidilactici) supplementation on performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acid profile in laying hens. *Poult Sci* 91, 2691-2700 (2012).

14. E. P. o. Additives, P. o. S. u. i. A. Feed, Scientific Opinion on Bactocell PA 10 (Pediococcus acidilactici) as a feed additive for laying hens. *EFSA Journal* 8, 1865 (2010).

15. E. P. o. Additives, P. o. S. u. i. A. Feed, Scientific Opinion on the safety and efficacy of Bactocell (Pediococcus acidilactici) as a feed additive for use in water for drinking for weaned piglets, pigs for fattening, laying hens and chickens for fattening. *EFSA Journal* 10, 2776 (2012).

ASSESSMENT OF PROTECTION INDUCED BY INACTIVATED TRIVALENT VARIANT REOVIRUS VACCINE AGAINST CHALLENGE WITH CONTEMPORARY REOVIRUS FIELD ISOLATES

M. Markis[A], A. Desmond[A], B. Ford[B], and M. Putnam[B]

[A] AviServe LLC, Newark, DE 19711
[B] Ceva Animal Health, Lenexa, KS

SUMMARY

Reovirus infections and associated diseases continue to be economically significant for U.S. broiler chicken producers. Currently the most important reovirus-associated disease in broiler chickens is viral arthritis/tenosynovitis (VA). The disease was successfully controlled through breeder vaccination since the 1980s, however the emergence of antigenic variant reoviruses around 2011 rendered commercial vaccines ineffective. Currently, the broiler industry utilizes autogenous reovirus vaccines to control VA. Multiple reovirus serotypes exist and continue to evolve over time, which complicates development of autogenous and commercial licensed vaccines. An inactivated reovirus vaccine containing three different antigenic variant reoviruses was developed and licensed in the United States. Protection against challenge with contemporary VA reovirus field isolates, both antigenically similar and distinct, was evaluated. Vaccination provided protection against antigenically related challenge reoviruses and reduced the severity of foot pad inflammation induced by some antigenically different challenge reoviruses. The cross-protective potential of the new commercial vaccine should help alleviate VA in broiler chickens.

INTRODUCTION

Avian reoviruses belong to genus *Orthoreovirus* in family *Reoviridae*. This family of viruses also includes *Rotavirus* genus and 28 other genera (2,3). Reoviruses are non-enveloped viruses with double capsids and segmented double-stranded RNA genomes. Reoviruses have been described in insects, plants, fungi, and most animals where they typically cause respiratory and enteric diseases. Avian reovirus infections are ubiquitous in poultry worldwide and have several disease presentations (15,18).

The most common and economically significant reovirus disease of poultry is viral arthritis and tenosynovitis (VA), which affects both chickens and turkeys (1,11,15,18). Reoviruses can be vertically transmitted from infected hens to progeny through eggs (5–7,10,15,20). Infected progeny can in turn transmit reovirus to susceptible contacts (9,15,20). Reoviruses can persist in immunologically sequestered sites of infected chickens for many weeks, and the virus can be shed incrementally over time (12).

Historically, VA was controlled in chickens through breeder vaccination with live and inactivated vaccines derived from a single serotype of reovirus, commonly referred to as S1133 (4,5,19,21). However, around 2011 VA re-emerged in broiler chickens due to a shift in antigenicity compared to the vaccine strains (13,14,16). Reoviruses isolated from chickens and turkeys since 2011 exist as multiple serotypes, genotypes, and pathotypes, which tend to change over time making disease control difficult (8,13,14,16,17).

Currently, poultry industry relies on vaccination of parent flocks with autogenous inactivated vaccines to diminish vertical transmission of variant reoviruses and provide progeny with maternal antibodies for early protection against reovirus infections. Reovirus isolates utilized in autogenous vaccines must be updated frequently to remain relevant, which can be challenging. A commercial inactivated reovirus vaccine containing three antigenic variant reovirus strains was developed recently. Efficacy of the inactivated trivalent variant reovirus vaccine in conjunction with a live prime vaccination was assessed against antigenically related and different contemporary reovirus challenge strains.

MATERIALS AND METHODS

Experimental design. Day-old Specific-Pathogen-Free (SPF) chicks were obtained from Charles River Laboratories. Upon arrival, all chicks were vaccinated with full doses of Vectormune® HVT IBD (Ceva Animal Health), SB-1 (Ceva Animal Health), and 89/03® (Merck Animal Health) via the subcutaneous route. Chicks were divided into three groups, one group of 105 chicks and two groups of 90 chicks. The group of 105 chicks, the reovirus

unvaccinated controls, were placed in a large colony house (LCH1) at day of age. The second and third groups of 90 chicks (vaccinates) were vaccinated subcutaneously with a full dose of V.A. ChickVac™ (Zoetis) and placed in two additional large colony houses (LCH2 and LCH3). At four weeks of age, all vaccinates were injected in right breast muscle with a full dose of inactivated trivalent variant reovirus vaccine (Ceva Animal Health). At eight weeks of age, one group of 90 vaccinates was injected in left breast muscle with a second full dose of inactivated trivalent variant reovirus vaccine. At 12 weeks of age, all chickens were challenged with contemporary variant reovirus isolates via the right foot pad route according to the experimental design in Table 1. Feed and water were provided *ad libitum*. Chickens were monitored daily, and foot pad inflammation assessed from 4 to 7 days post-challenge. Injection site reactions were also assessed during the necropsy.

Inactivated reovirus vaccine. Inactivated trivalent variant reovirus vaccine (Ceva Animal Health) contains three antigenic variant chicken reovirus strains, serotype 1/4455, serotype 2/4455, and serotype 3. The three reovirus serotypes are antigenically distinct from commercially available vaccine strains (S1133-like), and are commonly isolated reovirus serotypes from commercial broilers with viral arthritis and tenosynovitis in the United States. The vaccine is formulated in ENABL® adjuvant.

Reovirus challenge strains. Vaccinated and unvaccinated chickens were challenged with six different reovirus strains, at three challenge doses each ($10^{1.5}$, $10^{2.5}$, and $10^{3.5}$ $TCID_{50}$). One of the six strains was a homologous vaccine virus (AVS-JF, serotype 2/4455). Two of the six challenge strains were antigenically related to the vaccine strains based on virus neutralization assessment (AVS-THC and AVS-TCB8). Three of the six challenge strains were antigenically different from the vaccine strains based on virus neutralization assessment (AVS-THY, AVS-TOC, and AVS-PBB).

RESULTS

Chickens vaccinated either once or twice with the inactivated trivalent variant reovirus vaccine were protected against the homologous challenge virus (Table 2). Vaccination provided moderate protection against challenge viruses that were antigenically related to the vaccine strains based on virus neutralization assessments, including AVS-THC and AVS-TCB8 (Table 2). Vaccination did not prevent foot pad inflammation in all challenged birds but did significantly reduce the severity of inflammation compared to the challenge controls.

Vaccination with two doses of inactivated trivalent variant reovirus provided protection against a heterologous reovirus that was not antigenically related to the vaccine strains based on the initial virus neutralization assessment (AVS-PBB). Vaccination with a single dose of inactivated vaccine did not prevent foot pad inflammation in all birds but did moderately reduce the severity of foot pad inflammation compared to challenge controls (Table 2).

Vaccination did not provide protection against heterologous challenge viruses AVS-THY and AVS-TOC, however the severity of foot pad inflammation was moderately reduced at $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$ challenge doses for AVS-THY, and $10^{1.5}$, $10^{2.5}$, and $10^{3.5}$ $TCID_{50}$ challenge doses for AVS-TOC (Table 2).

Overall, vaccination with two shots of the inactivated trivalent variant reovirus vaccine induced better protection against reovirus challenge than vaccination with a single dose of the vaccine. No injection site reactions were observed in any of the chickens vaccinated with Ceva's inactivated trivalent variant reovirus vaccine nine weeks after the first vaccination and five weeks after the second vaccination.

DISCUSSION

Reovirus vaccination with live prime and inactivated trivalent variant reovirus vaccines elicited protection against the homologous challenge virus and against two antigenically related challenge strains, as expected. Additionally, vaccination provided protection against one antigenically different challenge virus, and resulted in reduced severity of inflammation caused by another two antigenically different challenge viruses. The cross-protection is likely achieved by the combination of live reovirus prime vaccination and three antigenic variant reovirus strains found in the inactivated vaccine. Reoviruses contain many epitopes against which antibodies are made. Some of these epitopes are group-specific while others are conserved among all avian reoviruses and can provide some limited cross-protection. The quantity of antibody produced post-vaccination is important to provide adequate protection and cross-protection against reovirus challenge. Finally, no injection site reactions were observed in chickens vaccinated once or twice with the inactivated trivalent variant reovirus vaccine.

REFERENCES

1. Dobson, K. N., and J. R. Glisson. Economic impact of a documented case of reovirus infection in broiler breeders. Avian Dis. 36: 788–791. 1992.

2. Fauquet, M.A.M., J. Maniloff, U. Desselberger, L. A. B. Genus Orthoreovirus. In: Virus Taxonomy, 1st ed. Elsevier Academic Press, San Diego, CA. 2005.

3. Fields, B.N., D.M. Knipe, P.M. Howley, D. E. G. Fields Virology 4th ed. Lippincott Williams and Wilkins, Philadelphia. 2001.

4. Giambrone, J. J., and R. P. Clay. Evaluation of broiler breeder pullet vaccination programs for preventing clinical reovirus infections in the progeny. Poult. Sci. 65: 457–461. 1986.

5. Giambrone, J. J., T. L. Hathcock, and S. B. Lockaby. Effect of a live reovirus vaccine on reproductive performance of broiler breeder hens and development of viral tenosynovitis in progeny. Avian Dis. 35: 380–383. 1991.

6. Hussian, M., P. B. S. Experimental Transmission of Avian Reovirus and Avian Adenovirus Through Embryonated Eggs. Aust. Vet. J. 57: 255–256. 1981.

7. Jones, R.C.,El-Taher, A. M. M. Reisolation of avian arthrotropic reovirus r2 from chicks infected as embryos. Avian Pathol. 14: 377–382. 1985.

8. Lu, H., Y. Tang, P. A. Dunn, E. A. Wallner-Pendleton, L. Lin, and E. A. Knoll. Isolation and molecular characterization of newly emerging avian reovirus variants and novel strains in Pennsylvania, USA, 2011-2014 OPEN. Nat. Publ. Gr. 5: 14727. 2015.

9. Markis, M. In vivo and in vitro characterization of virus isolates involved in viral enteritis/enteropathy, also known as runting stunting syndrome (RSS), in broiler chickens. ProQuest LLC, Ann Arbor, MI. 2014.

10. Menendez, N. A., B. W. Calnek, and B. S. Cowen. Experimental egg transmission of avian reovirus. Avian Dis. 19: 104–111. 1975.

11. Mor, S. K., T. A. Sharafeldin, R. E. Porter, A. Ziegler, D. P. Patnayak, and S. M. Goyal. Isolation and characterization of a Turkey arthritis reovirus. Avian Dis. 57: 97–103. 2013.

12. Roessler, D. E., and J. K. Rosenberger. In vitro and in vivo characterization of avian reoviruses. III. Host factors affecting virulence and persistence. Avian Dis. 33: 555–565. 1989.

13. Rosenberger, J.K., S.C. Rosenberger, M. Markis, J. P. R. Pathogenicity and control of recent (2011-2012) reovirus isolates from broiler and turkey flocks presenting with viral arthritis and tenosynovitis. American Association of Avian Pathologists, Chicago, IL. 2013.

14. Rosenberger, S.C., J.K. Rosenberger, M. Markis, J. P. R. Characterization of recent (2011-2012) reovirus isolates from broiler and turkey flocks presenting with viral arthritis and tenosynovitis using in vitro antibody dependent virus microneutralization assays. American Association of Avian Pathologists, Chicago, IL. 2013.

15. Rosenberger, J. K. Reovirus Infections. In: Diseases of Poultry. B. W. Calnek, ed. Iowa State University Press, Ames, IA. pp. 283–298. 2003.

16. Sellers, H. S. Current limitations in control of viral arthritis and tenosynovitis caused by avian reoviruses in commercial poultry. Vet. Microbiol. 206: 152–156. 2017.

17. Sharafeldin, T. A., S. K. Mor, A. Z. Bekele, H. Verma, S. M. Goyal, and R. E. Porter. The role of avian reoviruses in turkey tenosynovitis/arthritis. Avian Pathol. 43: 371–378. 2014.

18. Vanderheide, L. Viral arthritis/tenosynovitis: A review. Avian Pathol. 6: 271–284. 1977.

19. Vanderheide, L. The history of Avian reovirus. Avian Dis. 44: 638–641. 2000.

20. Vanderheide, L., and M. Kalbac. Infectious tenosynovitis (viral arthritis): characterization of a Connecticut viral isolant as a reovirus and evidence of viral egg transmission by reovirus-infected broiler breeders. Avian Dis. 19: 683–688. 1975.

21. Vanderheide, L., and R. K. Page. Field experiments with viral arthritis/tenosynovitis vaccination of breeder chickens. Avian Dis. 24: 493–497. 1980.

Table 1. Experimental design.

Treatment Group	Total Birds	Large Colony House	Reovirus Challenge Dose	Challenge Viruses						Unvaccinated/ Unchallenged Controls
				AVS-JF[A] Homologous	AVS-THC[A]	AVS-TCB8[A]	AVS-THY[B]	AVS-TOC[B]	AVS-PBB[B]	
				# Birds	# Birds	# Birds	# Birds	# Birds	# Birds	# Birds
Unvaccinated	105[C]	LCH1	$10^{1.5}$ $TCID_{50}$	5	5	5	5	5	5	5
			$10^{2.5}$ $TCID_{50}$	5	5	5	5	5	5	
			$10^{3.5}$ $TCID_{50}$	5	5	5	5	5	5	
V.A. ChickVac™ + 1 Shot Inactivated Trivalent Variant Reovirus Vaccine	90	LCH2	$10^{1.5}$ $TCID_{50}$	5	5	5	5	5	5	5
			$10^{2.5}$ $TCID_{50}$	5	5	5	5	5	5	
			$10^{3.5}$ $TCID_{50}$	5	5	5	5	5	5	
V.A. ChickVac™ + 2 Shots Inactivated Trivalent Variant Reovirus Vaccine	90	LCH3	$10^{1.5}$ $TCID_{50}$	5	5	5	5	5	5	5
			$10^{2.5}$ $TCID_{50}$	5	5	5	5	5	5	
			$10^{3.5}$ $TCID_{50}$	5	5	5	5	5	5	

[A] Isolate is neutralized *in vitro* by antisera made against the inactivated trivalent variant reovirus vaccine + V.A. ChickVac™.
[B] Isolate is NOT neutralized *in vitro* by antisera made against inactivated trivalent variant reovirus vaccine + V.A. ChickVac™.
[C] At 12 weeks of age, 5 birds were moved from LCH1 to LCH2 and 5 birds to LCH3 as unvaccinated/unchallenged controls.

Table 2. Protection induced by V.A. ChickVac™ prime and Ceva's inactivated trivalent variant reovirus vaccine against challenge with contemporary reovirus field isolates. SPF chicks were unvaccinated, vaccinated with V.A. ChickVac™ and a single dose of inactivated trivalent variant reovirus vaccine, or vaccinated with V.A. ChickVac™ and two doses of inactivated trivalent variant reovirus vaccine. AVS-JF reovirus isolate is included in the inactivated reovirus vaccine, and is the homologous challenge virus.

Challenge ID Serotype	Challenge Dose	Unvaccinated		V.A. ChickVac™ + 1 Shot of Inac. Reo Vac.[a]		V.A. ChickVac™ + 2 Shots of Inac. Reo Vac.[b]	
		Aff/Tot[c]	Mean Lesion Score[d]	Aff/Tot[c]	Mean Lesion Score[d]	Aff/Tot[c]	Mean Lesion Score[d]
AVS-JF[e] (homologous)	$10^{1.5}$ $TCID_{50}$	2/5	0.4	0/5	0.0	0/5	0.0
	$10^{2.5}$ $TCID_{50}$	5/5	3.0	0/5	0.0	0/5	0.0
	$10^{3.5}$ $TCID_{50}$	5/5	5.4	2/5	0.8	1/5	0.4
AVS-THC[e]	$10^{1.5}$ $TCID_{50}$	4/5	1.8	0/5	0.4	1/5	0.2
	$10^{2.5}$ $TCID_{50}$	4/5	1.4	2/5	1.0	0/5	0.0
	$10^{3.5}$ $TCID_{50}$	5/5	5.8	3/5	1.4	4/5	1.6
AVS-TCB8[e]	$10^{1.5}$ $TCID_{50}$	0/5	0.0	0/5	0.0	1/5	0.4
	$10^{2.5}$ $TCID_{50}$	3/5	1.2	3/5	1.4	3/5	1.4
	$10^{3.5}$ $TCID_{50}$	5/5	5.0	4/5	1.8	2/5	1.8
AVS-THY[f]	$10^{1.5}$ $TCID_{50}$	4/5	4.6	2/5	1.4	2/5	1.6
	$10^{2.5}$ $TCID_{50}$	5/5	5.6	5/5	2.9	5/5	4.0
	$10^{3.5}$ $TCID_{50}$	5/5	6.8	5/5	6.0	5/5	7.4
AVS-TOC[f]	$10^{1.5}$ $TCID_{50}$	3/5	1.2	1/5	0.4	0/5	0.4
	$10^{2.5}$ $TCID_{50}$	3/5	1.8	4/5	1.0	2/5	1.2
	$10^{3.5}$ $TCID_{50}$	5/5	4.4	4/5	2.0	3/5	1.0
AVS-PBB[f]	$10^{1.5}$ $TCID_{50}$	2/5	0.8	2/5	0.4	0/5	0.0
	$10^{2.5}$ $TCID_{50}$	5/5	2.2	2/5	1.2	0/5	0.0
	$10^{3.5}$ $TCID_{50}$	5/5	5.4	4/5	1.8	0/5	0.0
Unchallenged	None	0/4	0.0	0/5	0.0	0/5	0.0

[a] SPF chicks were vaccinated subcutaneously with V.A. ChickVac™ at day-of-age, and intramuscularly (breast) with Ceva's inactivated trivalent variant reovirus vaccine at four weeks of age.
[b] SPF chicks were vaccinated subcutaneously with V.A. ChickVac™ at day-of-age, and intramuscularly with Ceva's inactivated trivalent variant reovirus vaccine at four and eight weeks of age.
[c] Foot pads were evaluated for inflammation daily from four to seven days post-challenge. Chicken is considered "affected" if inflammation was observed at any time during the evaluation.
[d] A value was assigned for severity of foot pad inflammation with none (-) being 0, modest inflammation (+/-) being 1, and moderate-severe inflammation (+) being 2. A sum of daily values was divided by the number of birds (5) per treatment group to enumerate the severity of inflammation.
[e] Reovirus isolate is neutralized *in vitro* by antisera made against Ceva's inactivated trivalent variant reovirus vaccine + V.A. ChickVac™.
[f] Reovirus isolate is NOT neutralized *in vitro* by antisera made against Ceva's inactivated trivalent variant reovirus vaccine + V.A. ChickVac™.

DEVELOPMENT OF A RAPID METHOD FOR WHOLE GENOME SEQUENCING OF FOWL ADENOVIRUSES

J. McElreath[A], D. Moormeier[A], J. Goza[A], C. Tobaben[A], J. El-Attrache[A], and S. Callison[A]

[A]Ceva Animal Health, Lenexa, KS 66215

SUMMARY

Fowl adenoviruses (FAdV) are widespread throughout all avian species and associated with myriad diseases in poultry (1). Inclusion body hepatitis (IBH) is an economically significant disease throughout the world caused by multiple serotypes of FAdV (2). It is common practice in the poultry industry to submit samples from suspect IBH cases for FAdV detection, isolation, and/or genetic characterization, all of which aid in determining the most appropriate preventative/protective measures to be taken for poultry flocks. Therefore, we sought to develop a rapid and cost-effective method for whole genome sequencing of FAdV from field samples. By combining novel lab protocols, nanopore-based DNA (deoxyribonucleic acid) sequencing, and custom bioinformatic tools, we can sequence, assemble, and analyze complete FAdV genomes from as many as 96 samples in approximately four hours. The genomic information can be used to unambiguously type FAdV that are present in field samples, including if more than one virus exists in a particular sample.

MATERIALS AND METHODS

Nucleic acid was extracted from liver tissue and cell culture samples sourced from various broiler farms. Liver tissue was prepared by manually homogenization in phosphate buffered saline (PBS). The Zymo Research HostZERO™ Microbial DNA kit was used to purify and extract viral DNA while also minimizing host DNA. After purification, fowl adenovirus DNA was quantified using the real-time PCR assay described by Günes *et al*. (3), and host DNA was quantified using the chicken alpha collagen qPCR assay (assay sequences are listed in Table 1).

DNA sequencing libraries were prepared using the Rapid Barcoding kits from Oxford Nanopore Technologies (ONT); for ≤12 samples, the Rapid Barcoding kit was used, and for 13-96 samples, the Rapid Barcoding kit 96 was used. The DNA libraries were sequenced using an ONT flow cell (version R9.4.1) and a GridION Mk1 or MinION Mk1B.

Sequence data was analyzed using proprietary software and workflows developed by Ceva Animal Health; this was accomplished by using Geneious Prime (version 2022.0.2) bioinformatics software.

RESULTS

Fowl adenovirus-specific qPCR resulted in detectable viral loads in both tissue and cell culture samples. Additionally, host (chicken) DNA was detected in host-specific qPCR. Despite a high amount of host reads in the sequence data, accurate typing of all fowl adenovirus was accomplished using proprietary software and workflow within Geneious Prime. Typing was confirmed by comparison to short-read sequence data performed by a separate internal laboratory.

CONCLUSIONS

This cost-effective process of DNA extraction, library preparation, sequencing, and data analysis allows for accurate typing of fowl adenovirus within four hours. Additionally, the whole genome is sequenced and PCR bias is eliminated. Rapid sequencing and typing can improve the sample turnaround time in both research and diagnostic laboratories, aiding in vaccine development and disease prevention.

REFERENCES

1. Hess, M. Aviadenovirus Infections. In: Diseases of poultry, 14th ed. D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. Nair, eds. Wiley-Blackwell Publishing Hoboken, NJ. pp. 322 –332. 2013.

2. Schachner, A., M. Matos, B. Grafl, and M. Hess. Fowl adenovirus-induced diseases and strategies for their control – a review on the current global situation. Avian Pathology. 47:2:111-126. 2018.

3. Günes, A., A. Marek, B. Grafl, E. Berger, and M. Hess. Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). J. of Vir. Methods. 183:2:147-153. 2012.

Table 1. Sequences of quantitative PCR assays for detection of fowl adenovirus and host (chicken) DNA.

Assay	Oligo name	Oligo Sequence (5'->3')
Günes Adenovirus DNA	52k-fw	ATGGCKCAGATGGCYAAGG
	52k-rv	AGCGCCTGGGTCAAACCGA
Alpha collagen chicken DNA	Forward primer	GGGAACTGGAGAACCCAATTTT
	Reverse primer	CGTGCCGCTGTCTCTACCAT
	Probe	CCCTTAACTGAGTTCCCCAGCTACTGCAG

CONCOMITANT MONITORING OF *CLOSTRIDIUM PERFRINGENS* AND *EIMERIA* SPECIES IN FECAL SAMPLES OF CONVENTIONAL AND SLOW-GROWING BROILER FLOCKS BY SCREENFLOX® MULTIPLEX QPCR WORKFLOW

E. Igwe[A], T. Van Limbergen[D], M. Dargatz[B], F. Thiemann[A], N. Dusch[B], N. Kryzhevoy[A], and A. Menconi[C]

[A]Evonik Operations GmbH, Germany. Rodenbacher Chaussee 4, 63457 Hanau-Wolfgang, Germany
[B]Evonik Operations GmbH, Germany. Kantstrasse 2, 33790 Halle (Westfalen), Germany
[C]Evonik Corporation, 1701 Barrett Lakes Blvd., Suite 340, Kennesaw, GA 30144 USA
[D]Pehestat BV, Dwarsstraat 5, 3560 Lummen, Belgium

SUMMARY

Necrotic enteritis infection by *Clostridium perfringens* (*Cp*) alone is well characterized, but the co-infection with *Eimeria* spp. is not fully elucidated. Herein, a survey to evaluate the prevalence of *Cp* by detection of toxin A and *Eimeria* spp. in pooled samples from conventional and slow-growing broiler flocks across 45 farms was conducted. The prevalence of *Cp* was significantly higher (79/88; 90%) for samples from slow-growing flocks than for conventional flocks (61/155; 39%). The most prevalent *Eimeria* spp. in conventional and slow-growing broilers were *E. acervulina* (98/155; 63%) and *E. maxima* (49/88; 56%) respectively. In contrast, *E. tenella* was the least prevalent in samples from the conventional (39/155; 25%) and the slow-growing flocks (9/88; 10%). Interestingly, the prevalence of *Cp* and *Eimeria* spp. increased between 7 and 28 days-of-age for both flocks. These findings further underline a potential interaction of *Cp* and *Eimeria* in the pathogenesis of enteric diseases in broilers.

INTRODUCTION

Strains of *Clostridium perfringens* (*Cp*) have been widely isolated from environmental and intestinal samples of broiler chicken flocks evaluated as healthy (1,2). The onset of clinical manifestation of necrotic enteritis (NE) has been associated with the overgrowth of pathogenic strains of *Cp* and the production of toxins, especially the netB toxin (2,3,4). The characterization and quantification of toxins produced are key in the process of elucidating the, so considered, normal and overgrowth levels of *Cp* and the etiology of NE. Furthermore, the pathogenesis of *Cp* and the interaction with *Eimeria* spp. infection as predisposing factor to NE in broiler chickens have been documented from different perspectives, including commercial field evaluations and disease models (5,6,7,8). Though such interaction has been studied, there is still some knowledge gaps on the prevalence of the different species of *Eimeria* and their interactions with toxins produced by *Cp*. Attempts aimed at closing such gaps might lead to a better resolution of the infection window before and during the clinical signs of NE; thus, expanding the ability of broiler producers to implement preventive and control measurements. Therefore, the objective of this study was to evaluate the use of a microbial monitoring qPCR workflow capable of detecting and quantifying the three endemic *Eimeria* species (*Eimeria acervulina*, *Eimeria maxima*, *Eimeria tenella*) in broiler chicken flocks, as well as alpha and *netB* toxins of *C. perfringens*.

MATERIALS AND METHODS

Sample collection. Fecal samples from commercial broiler flocks under two different production management systems (45 poultry farms across Belgium) were collected for this study. Ross 308 birds were raised in 39 of the farms by conventional production and slow-growing birds in six farms. One house per farm was sampled for the conventional farms, while two to three houses were sampled for the farms with slow-growing birds. Representative samples were collected on days 7, 14, 21, 28, and 40 for the conventional broilers and on days 14, 28, 60-63, and 67-70 for the slow-growing broilers. Through-out this trial, a total of 155 pooled samples were collected from the conventional broilers and 88 pooled samples were collected from the organic farms. At each collection time point or event, 96 individual samples were picked up from each house and pooled in a plastic sample collection bag, while walking through the house in a zig-zag pattern. Samples were homogenized for 3-5 minutes; then, 5 grams was transferred to ScreenFloX® PCR sample collection tubes. Samples were pretreated and DNA extraction was conducted with ScreenFloX® PCR DNA Extraction kit.

DNA Quantification. For the detection and quantification of *Clostridium perfringens* (Toxin A gene (cpa) and netB) and *Eimeria* spp. (*E. tenella*, *E. maxima,* and *E. acervulina*) a multiplex qPCR for both pathogens were performed with ScreenFloX® PCR detection and quantification kits, following the manufacturer's instructions. In brief, 20 µl master mix consisting of 5 µl Master A, 15 µl master B and 1 µl of IC (internal control) was prepared according to the instruction of the manufacturer. 20 µl of the master mix were dispensed into individual wells of a 96 well plate. Then, a 10 µl of the extracted DNA sample was transferred into each well. 10 µl of the respective standard and 1 µl of IC were transferred to each standard well accordingly. The contents of the plate were mixed thoroughly with a multi-channel pipet, and the plate was sealed with a Clear Weld Seal Mark II foil film. The plate was centrifuged for 30 seconds at 1000 g (~3000 rpm). Finally, the plate was run on a CFX96 real time PCR instrument (Bio Rad, Germany) with the following PCR conditions: 45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 45 seconds and extension at 72°C for 15 seconds. The quantification of markers in samples were determined from the standard curve constructed with standard known concentrations of gene targets of pathogens to be quantified.

RESULTS AND CONCLUSION

Prevalence of *C. perfringens* and *Eimeria* species in conventional and slow-growing broilers. The overall prevalence of *C. perfringens*, both netB harboring (cpa+ netB+) and netB negative (cpa+ netB-) strains, as well as the prevalence of *Eimeria* species (*E. tenella*, *E. acervulina* and *E. maxima*) in all pooled fecal samples collected from the conventional and slow-growing flocks is shown in Figure 1. The rates of detection of cpa and netB in slow-growing flocks (79 out of 88 (90%) and 72 out of 88 (82%) respectively) were significantly higher than the rates of the detection of both markers (61 out of 155 (39%) and 3 out of 155 (1.9%) for cpa and netB respectively) in conventional flocks ($p < 0.01$ for both cpa and netB). The three *Eimeria* species tested for in this study were detected in samples from the slow growing and conventional flocks; the recovery rate of *E. tenella* was the lowest for both flock types. From all samples obtained from the conventional flocks, the detection rates of *E. acervulina*, *E. maxima* and *E. tenella* were 63% (98 out of 155), 43% (66 out of 155) and 25 % (39 out of 155) respectively. While the detection rates of *E. acervulina*, *E. maxima* and *E. tenella* were 41% (36 out of 88), 56% (49 out of 88) and 10% (9 out of 88) respectively in samples obtained from the slow-growing flocks. The higher rates of detection of *E. acervulina* and *E. tenella* obtained for the conventional flocks were statistically significant ($p < 0.01$) for *E. acervulina* and $p < 0.01$ for *E. tenella*) compared to the detection rates of the slow-growing flocks. In contrast, the higher rate of detection of E. maxima in the slow-growing flocks was statistically significant ($p < 0.01$).

Necrotic enteritis outbreak in slow-growing flocks and prevalence of *C. perfringens* and *Eimeria* species. Eleven out of 17 slow-growing flocks (64.7%) had necrotic enteritis (NE) outbreak during this study, whereas 35.3% were NE negative through-out sampling. Interestingly, most of the houses in the NE outbreak farms were NE positive (range: 66.7-100%); and in the same pattern, most of the houses of NE- negative farms were NE- negative. As an attempt to investigate the prevalence of *C. perfringens* and *Eimeria* species during the diagnosed period of necrotic enteritis, samples collected at the window of occurrence/ NE-diagnosis (14 – 28 day) from NE positive and NE negative flocks of the slow growing flocks were compared (Table1B). The rates of detection of cpa and netB in NE positive flocks were (38 out of 42 (90%) and 33 out of 42 (79%) respectively) higher than the rates of detection (12 out of 15 (80%) and nine out of 15 (60%) in NE- negative flocks. Despite the higher rates of detection of both targets in NE positive flocks, only the prevalence of netB was statistically significant ($p < 0.01$). The rates of detection of the three *Eimeria* species evaluated in this analysis were not statistically significant (Table 1 B) when compared for NE positive and NE-negative flocks.

Influence of the administration of coccidia vaccine or coccidiostats on the recovery of *C. perfringens* strains and *Eimeria* species in conventional flocks. Since two types of coccidiosis control programs were used by the conventional producers enrolled in this study, we decided to compare the recoveries of *C. perfringens* and *Eimeria* species. A subset of samples was obtained from five flocks that were vaccinated versus 35 flocks that were not vaccinated and treated with coccidiostats. Interestingly, the rate of detection of cpa and netB genes of *C. perfringens* in samples from the non-vaccinated flocks were higher than those of the vaccinated flocks; however, the differences in the detection rates of both markers for both groups were not statistically significant (Table 1A). In contrast, the rates of detection of all three species of *Eimeria* were higher for samples obtained from the vaccinated flocks (25%, 60% and 95% for *E. tenella*, *E. acervulina*, and *E. maxima* respectively) compared to those of the non-vaccinated flocks (19%, 55% and 27% for *E. tenella*, *E. acervulina*, and *E. maxima* respectively). However,

only the detection rate of *E. maxima* was statistically significant (P <0.01) when compared for the vaccinated and the non-vaccinated flocks.

ACKNOWLEDGEMENTS

The authors would like to thank all farms, integrators, and farm-veterinarians for collaborating with us in this study.

REFERENCES

1. Craven S., N. Stern, J. Bailey, N. Cox. Incidence of *Clostridium perfringens* in broiler chickens and their environment during production and processing. Avian Dis. 45:887–896; 2001.

2. Opengart, K. 2008. Necrotic Enteritis. Diseases of Poultry, 12th ed., Y. M. Saif, A. M. Fadley, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, ed. Wiley-Blackwell Publishing, Ames, Iowa, pages: 872–879.

3. Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol. 17(1):32-36. 2009.

4. Timbermont, L., F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol. 40:341-347. 2011.

5. Palliyeguru, M. W. C. D., and S. P. Rose. Sub-clinical necrotic enteritis: its aetiology and predisposing factors in commercial broiler production. World's Poultry Sci. J. 70(4):803-816. 2014.

6. Prescott, J. F., J. A. Smyth, B. Shojadoost, and A. Vince. Experimental reproduction of necrotic enteritis in chickens: a review. Avian Pathol. 45:317-322. 2016.

7. Smyth, J. A. Pathology and diagnosis of necrotic enteritis: is it clear-cut? Avian Pathol. 45:282-287. 2016.

8. Moore, R. J. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathol. 45:275-281. 2016.

Figure 1. Prevalence of *C. perfringens* strains detected by toxin A (cpa) and *netB* genes; and *Eimeria* species (*E. acervulina, E. tenella and E. maxima*). White bars represent the prevalence of *C. perfringens* (*cpa and netB* genes) and *Eimeria species* in % for the conventional flocks, while the black bars represent the prevalence of *C. perfringens* (*cpa and netB genes*) and *Eimeria species* in % for the slow growing flocks. * on bars represent groups with significant differences at $p < 0.05$ (determined by two-tailed Fisher exact test).

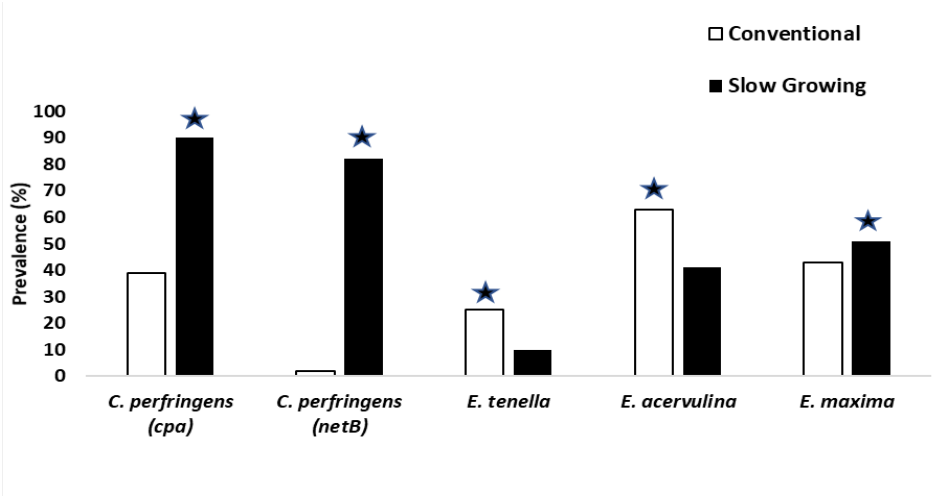


Table 1. A. Prevalence of *C. perfringens* and *Eimeria* species in samples collected at the window of 14 to 28 days of necrotic enteritis outbreak and NE- negative flocks of slow-growing farms. B. Effect of the administration of coccidia vaccine or coccidiostats on the recovery of *Clostridium perfringens* and *Eimeria* spp. in conventional flocks. * represent groups with significant differences at $p < 0.05$ (determined by two-tailed Fisher exact test).

	A: % detection rate of pathogens in pooled samples of NE positive and negative slow- growing flocks			**B: % detection rate of pathogens in pooled samples of vaccinated and non-vaccinated conventional flocks**		
Pathogens	Necrotic Enteritis	NE-Negative	P-value	Vaccinated	Non-vaccinated	P-value
C. perfringens (*cpa*)	38/42 (90%)	12/15 (80%)	0.116	3/15 (15%)	38/96 (40%)	0.192
C. perfringens netB	33/42 (79%)	9/15 (60%)	0.018*	0/15 (0%)	1/96 (1.0%)	> 0.999
E. tenella	4/42 (10%)	2/15 (13%)	0.649	5/20 (25%)	18/96 (19%	0.633
E. acervulina	14/42 (33%)	4/15 (27%)	0.417	12/20 (60%)	53/96 (55%)	0.843
E. maxima	27/42 (64%)	10/15 (57%)	0.856	19/20 (95%)	26/96 (27%)	< 0.01*

FINE-TUNING OF AN INFECTIOUS BRONCHITIS VACCINATION PROGRAM IN LAYERS EXPOSED TO THE DMV/1639 STRAIN

A. Mendoza-Reilley[A], B. Jordan[B], H. Sellers[B], and R. Gallardo[C]

[A]Merck Animal Health, DeSoto, KS, 66018
[B]The University of Georgia, Athens, GA, 30602
[C]UC Davis, School of Veterinary Medicine, CA, 95616

INTRODUCTION

Infectious bronchitis virus (IBV) is an economically significant pathogen of poultry. Infection with IBV results in a mild respiratory infection, and in broilers predisposes chickens to secondary bacterial infections leading to airsacculitis and condemnation at processing. For broiler-breeders and commercial egg layers, disease is most often associated with the reproductive tract and can range from false layer syndrome and cystic oviduct development to sharp decreases in egg production and quality to inapparent infection.

In 2011, a nephropathogenic IBV strain was characterized from three broiler flocks located in Delmarva and was classified as DMV/1639/11 (1). From 2015 forward, the DMV/1639 strain has progressed to strictly respiratory disease in broilers and has spread across the US. It has also been associated with false layer syndrome in commercial layers and decreases in egg production in broiler breeders. As of this writing, the DMV/1639 strain is the most significant variant IBV affecting commercial poultry across all sectors.

Because of the economic significance for both meat and egg type bird production, nearly all commercial poultry are vaccinated against the disease. One major challenge to achieving protection, however, is that different serotypes of IBV do not confer cross-protection making current vaccines less effective against novel IBV variants like DMV/1639. In an attempt to induce a level of cross-protection and to mitigate the clinical signs and negative effect on egg production, table egg producers have modified their standard vaccination program by using multiple IBV serotype vaccines and adjusting the vaccination schedule to achieve earlier protection in the flock.

A second major challenge for protection from disease achieved by IBV vaccination is ensuring that vaccines are adequately applied (2). A major focus on hatchery vaccine application has occurred in the broiler industry, but the understanding of vaccine application and infection and replication dynamics in broiler breeders and commercial layers is less defined and understood. This can be monitored in two ways; through quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR; "real-time PCR") to measure viral loads and serology to assess the immune response after infection.

Herein we report a process for analyzing vaccine efficacy and field challenge for IBV in a commercial layer facility using these tools that can be applied across the layer and broiler breeder industries.

SUMMARY

After several field cases reported as false layer syndrome (FLS) in 2017, table egg producers implemented the use of a live attenuated IBV vaccine at the hatchery or within the first five days of age via spray. ELISA and HI serology testing were performed, in addition to periodic mortality and bird health assessments, as part of the company surveillance program. Additional testing through real-time PCR at different times after vaccination was also implemented to collect information on vaccination coverage and exposure to IBV DMV/1639 strain.

The objective of this report is to show the process of refining the existing IBV vaccination programs of commercial egg producers located in the United States, to mitigate clinical lesions and egg production issues caused by the IBV variant DMV/1639.

MATERIAL AND METHODS

Companies interested in controlling potential IBV variants affecting the flocks with FLS or drops in egg production would choose one or two flocks of their preference to perform an assessment of IBV in the pullets throughout production. The surveillance program should start with early evaluation of tracheas or choanal swabs at seven days post vaccination, followed by 3, 6, 10, and 16 weeks of age sampling times (times may be adjusted based on vaccine schedule). Serum samples would be added to the sampling protocol at 10 and 16 weeks of age. The

surveillance program provides more significant information by extending it to the flock in production and sampling the same tissues every 10-12 weeks through the end of the cycle, so this is also recommended. Vaccination programs from different regions in the United States can then be assessed through the data gathered using this surveillance program.

DISCUSSION

Results and correlation of findings throughout the surveillance process confirmed the use of Massachusetts type vaccines such as Mildvac-Ma5 at early age (3). This is expected as these vaccines are widely used in the layer and broiler industry to reduce the negative effects of IBV variants. The use of Ma5 vaccine has been reported to be effective in protecting against early challenge with IBV strains of different serotypes, by assessing ciliary activity of the tracheal epithelium (4). Furthermore, the variant IBV DMV/1639 and novel PA/1220-like strains were identified during this exercise, suggesting that FLS and decreases in egg production after the peak could be related to appearance of these variants in the egg production systems. These field cases and surveillance support previous findings that age at the time of infection is the major factor in the development of FLS in laying hens (5).

Although attenuated live vaccines have not been able to completely prevent or eliminate the circulation of IBV strains associated with FLS, adjusting the IBV vaccination program has reduced the FLS-related clinical signs. Other egg production drops have been observed, variants have been identified, and vaccine application quality has become a critical area to improve to better immunize the birds, and to bring a broader protection against different IBV strains.

REFERENCES

1. J. Gelb et al (2005) Avian Pathology, 34 (3), 194-203.

2. Jordan (2017) Veterinary Microbiology, 206, 137-143.

3. A.P da Silva et al. (2021) Avian Diseases, 65 (3), 373-380.

4. J. Cook et al (1999) Avian Pathology 28, 477-485.

5.Mueller-Slay (2021) American Association of Avian Pathologists Meeting, https://aaap.memberclicks.net/assets/2021_Annual_Meeting/AAAP_2021_Proceedings.pdf.

Figure 1. Timeline of appearance of FLS Commercial Layers in the United States and Canada.

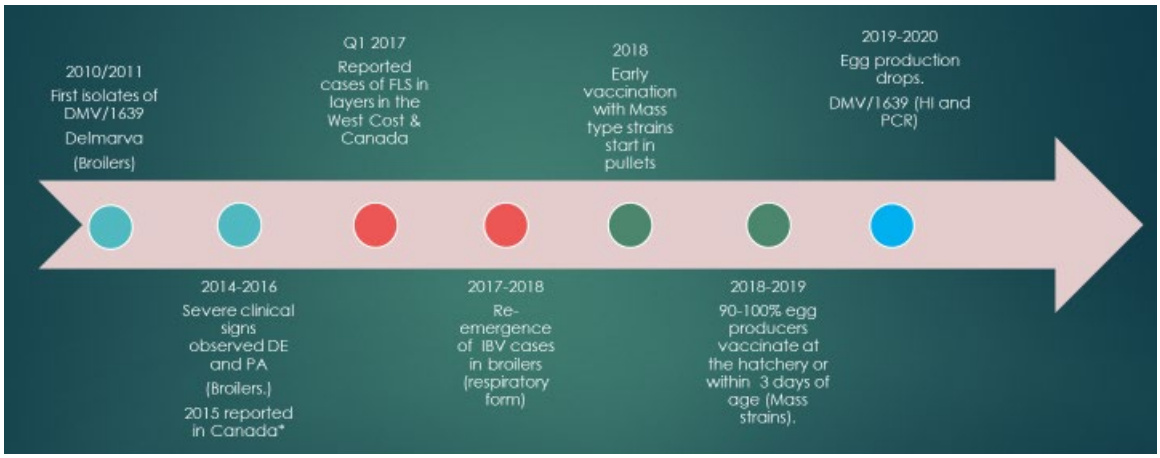


Table 1. Example of a tissue tracheal tissue survey for IBV panel (source. Dr. Brian Jordan Laboratory UGA).

Sample Name	Target	Ct	Target	Ct	Target	Ct	Target	Ct	Target	Ct	Target	Ct	Target	Ct	Target	Ct
Pullet 1 Tr	Generic IBV	30.8693352	Ga08	Negative	DMV/1639	29.62537	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative
Pullet 1 Tr	Generic IBV	25.6492596	Ga08	Negative	DMV/1639	31.18489	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative
Pullet 1 Tr	Generic IBV	31.1962051	Ga08	Negative	DMV/1639	29.99474	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative
Pullet 1 Tr	Generic IBV	26.6506023	Ga08	Negative	DMV/1639	30.51495	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative
Pullet 1 Tr	Generic IBV	30.8603745	Ga08	Negative	DMV/1639	30.08314	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative
Pullet 1 Tr	Generic IBV	26.1004486	Ga08	Negative	DMV/1639	28.78996	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative
Pullet 1 Tr	Generic IBV	30.9469948	Ga08	Negative	DMV/1639	31.42771	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative
Pullet 1 Tr	Generic IBV	35.8056297	Ga08	Negative	DMV/1639	34.08781	Mass	Negative	Ark	Negative	GA13	Negative	Del072/GA98	Negative	Conn	Negative

FIRST SEROEPIDEMIOLOGICAL SURVEY OF AVIAN METAPNEUMOVIRUS CIRCULATION IN MOROCCAN BROILER FARMS

A. Mernizi[A,B,*], S. Bouziane[B], H. Fathi[A], J.L. Criado[A], M. Bouslikhane[C], A. Ghram[D], E. Catelli[E], M. Mouahid[F], and S. Nassik[B]

[A] HIPRA Laboratorios S.A., 17170 Av. Selva, Amer, Spain
[B] Département de Pathologie et Santé Publique Vétérinaire, Unité de Pathologie Aviaire, IAV Hassan II, Rabat, Morocco
[C] Département de Pathologie et Santé Publique Vétérinaire, Unité de Microbiologie, Immunologie et Maladies Contagieuses, IAV Hassan II, Rabat, Morocco
[D] Laboratoire d'Epidémiologie et de Microbiologie Vétérinaire, Institut Pasteur de Tunis, Université Tunis El Manar, Tunisia
[E] Department of Veterinary Medical Sciences – University of Bologna, Italy
[F] Cabinet vétérinaire Mouahid, Témara, Morocco

*Corresponding author: amine.mernizi@hipra.com

SUMMARY

Although avian metapneumovirus (aMPV), a highly contagious respiratory virus for poultry, is causing significant economic losses in broilers, the epidemiological aspects of the virus circulation and its distribution are still poorly understood in Morocco.

Given that, we carried out a pilot seroepidemiological survey from December 2020 to June 2021 to define the seroprevalence of aMPV infection in Moroccan broiler farms. The survey concerned different bioclimatic areas of Morocco (humid to subhumid, semi-arid, and arid bioclimate) between winter and spring.

Forty-eight farms strictly not vaccinated against aMPV were selected based on a stratified random sampling model from different bioclimatic areas. Then, sera were collected from fifteen to thirty birds of each farm following a simple random sampling, and analyzed by a commercial indirect ELISA kit (CIVTEST AVI TRT®, HIPRA S.A., Amer, Spain) able to detect and titer antibodies against both predominant subtypes A and B of aMPV.

Furthermore, questionnaires were shared with veterinarians in charge of the farms to collect zootechnical and sanitary data, which were analyzed later with multivariable logistic regression models, to identify risk factors involved in aMPV circulation.

From 1142 of total sera analyzed, 912 were tested positive to the ELISA, showing an overall aMPV seroprevalence of 79.86%. For winter and spring, the seroprevalences obtained for the humid to sub-humid bioclimatic areas were 90.63% and 67.69%, respectively. The seroprevalences in the semi-arid bioclimate were 62.46% and 88.62%, still, for winter and spring, respectively. The arid bioclimate recorded the highest seroprevalences: 94.16% during winter and 84.82% during spring. We could not collect samples to represent the hyper-arid bioclimate for logistical reasons.

The bioclimate and the season seem to contribute to aMPV circulation regarding risk factors. Likewise, high densities and poor hygiene standards are proven to predispose to the virus's presence.

Hereby we showed the circulation of avian metapneumovirus in Moroccan broiler farms, influenced by the bioclimate, the season, the farm management, and general hygiene conditions. The present is the first serological evidence of avian metapneumovirus in broilers in the whole Maghreb region.

INTRODUCTION

Avian metapneumovirus (aMPV) belongs to the subfamily of *Pneumovirinae*, within the large family of *Paramyxoviridae* (1). It causes the turkey rhinotracheitis (TRT) in turkeys, and, in broilers, the virus is considered a predisposing factor to the swollen head syndrome, alongside bacterial infection (2, 3). Known as SHS, this syndrome is up to date present in most areas of the world (4).

Although the common belief that avian metapneumovirus always triggers swollen head syndrome in chickens, aMPV mono-infected flocks may not be accompanied by clinical symptoms (5), or paradoxically, can express severe respiratory distress (6).

Four subtypes of aMPV have been identified so far. They are genetically and antigenically different and are named A, B, C, and D (7). The aMPV-A and aMPV-B (subtypes A and B) are more widespread globally, even though field evidence points out the higher circulation of subtype B viruses over subtype A worldwide for reasons still poorly understood (8 – 10).

Serology is a useful tool for aMPV diagnosis. In this regard, ELISA seems to be the most common way for that (11 – 13), particularly in unvaccinated flocks where seroconversion is a clear indicator of field virus contact with birds. Besides, many commercial ELISA kits detect subtypes A and B without differentiation, as both belong to a single and common serotype (14).

The chicken meat production in Morocco has noticed tremendous growth over time, jumping from an annual mean of 86.000 tons produced in the eighties to 535.000 tons in 2020. It represents an increase of more than six times in four decades (15), contributing significantly to the national animal-protein demand, although struggling with many handicaps.

Unfortunately, the live market still dominates the sector, with only 10% processed in slaughterhouses. Besides, due to the lack of lands and restrictive policies of the law 49-99, regulating the poultry industry, many producers had to look far away from traditional areas, keened for the good climate and proximity to large consumption centers, and to implement new farms in other regions less convenient, whereas 70% of the country is represented by thermal variations from “temperate” to “very hot” (16). Furthermore, the broiler segment suffers from the unavailability of highly qualified staff to ensure adequate management procedures standards (17). These aspects make the sector vulnerable, particularly to infectious diseases like avian metapneumovirus-induced disease.

Avian metapneumovirus in broilers in Morocco was first described during the eighties (18). No other studies have been published later on this issue, and aMPV field cases have often been reported without data on circulating subtypes.

In this respect, the purpose of the present survey was to investigate the presence of aMPV antibodies in serological naïve chickens to demonstrate the circulation of aMPV in broiler farms in Morocco for the first time. Moreover, each flock’s clinical, field, and biosecurity data were collected by interviewing poultry field veterinarians involved in the study. Data were analyzed along with aMPV serological results, bioclimatic areas where the farms were located and seasons, to identify the main risk factors for the aMPV occurrence.

MATERIAL AND METHODS

Sampling protocol. The survey covered the period from December 2020 to June 2021 and targeted broiler flocks not vaccinated against avian metapneumovirus and older than five weeks, an age that allows making evidence of any late seroconversion.

The sampling protocol was based on two steps to achieve the statistical representativity of the study: first, selecting the broiler farms with stratified random sampling, where each stratum (sub-group) represents a bioclimatic area (16)—, and second, choosing birds with a simple random selection from each farm.

Given the lack of data about the avian metapneumovirus in Morocco, the sample size was calculated for the number of flocks or sera hypostasizing a theoretical prevalence of 10%.

Flock selection. The farms were located in different geographical areas of Morocco and were sampled regardless presence of respiratory signs. The number of sites per bioclimate area was proportional to farm density in each one.

Starting from an initial population of 7992 broiler farms (ONSSA data, 2021), the number of flocks to be sampled were selected according to Pfeiffer (19) (with a confidence interval of 95%).

Based on that, the minimum number of flocks needed for the study was $n_1 = 29$ flocks. Nonetheless, we oversized this number and investigated 48 herds to compare the two seasons and bioclimatic areas.

According to the bioclimate areas present in Morocco, forty-eight flocks were randomly selected while respecting the proportion of farms authorized in each region as reported in the Table 1.

Sample size. The number of blood sera to be collected per flock was defined according to Pfeiffer (19) and was set to $n = 29$. Notwithstanding, for several logistic parameters (predefined number of samples taken in the field by veterinarians, or eventual losses during transport or storage), we lessened the sampling size in some cases up to fifteen.

Sample collection. Samples consisted of fresh blood collected from brachial wing veins during flock’s visits by puncture of the alar veins then stored in sterile tubes before transportation to the Avian Pathology Unit of IAV Hassan II Veterinarian school, Rabat, at +4°C. It should be pointed out that the time gap between finishing all the blood collection in one site and the refrigeration, the tubes were placed on a slanted surface and under room temperature, allowing clotting and initial separation of serum from coagulated blood.

Once at the Avian Pathology Unit of IAV Hassan II lab, sera were fully extracted and stored on Eppendorf tubes under -20°C, until the day of analysis.

Samples also consisted of prepared frozen sera sent by veterinarians for the flocks we did not visit.

For confidentiality purposes, a unique alphanumeric code was assigned to each flock.

Sample analysis. The sera were analyzed by the commercial indirect ELISA kit CIVTEST AVI TRT® (HIPRA S.A., Amer, Spain), allowing detection of aMPV-A and aMPV-B antibodies. The mean titers, validity tests, and coefficients of variation are automatically calculated by flock and sample series with the software HIPRASOFT® 5.0 (HIPRA S.A., Amer, Spain).

It is important to mention that all the samples were transported at +4°C and analyzed at the Avian Pathology Unit of IAV Hassan II Veterinarian school, Rabat.

Field data collection. Field and biosecurity parameters. Using the questionnaire assigned to veterinarians, information on field data and the measures applied for biosecurity were collected for flocks included in the aMPV serosurvey, except when veterinarians were reluctant to share inputs of herds not visited. The data accuracy of the survey was enhanced by ensuring conducting personal interviews on the farm whenever possible.

The different parameters concerned were: farm localization, age of the flock, density, single age or multi-age, ventilation system, "swollen head syndrome" antecedent outbreaks, vaccination program, as well as general hygiene and biosecurity applied, assessed using an adapted evaluation grid, to assign a biosecurity score to each flock, including litter quality. It is largely known that biosecurity and hygiene conditions of the farm are important risk factors for the occurrence of aMPV infection and SHS.

Besides, the GPS localization was recorded to check the distance from neighbouring poultry farms later whenever a flock was visited.

Exploitation of data. The inputs from questionnaires, the evaluation grid, and serology were gathered into a Microsoft® Excel® (2016) workbook. Those about the field respiratory diseases survey were summarized and processed separately, with the following attributions:

The frequency of observation was converted to numeric values, from 1 to 4, representing respectively: never observed, rare, often, very frequent;

The relevance in the field was also adapted to an ordinal score from 1 (the less relevant) to 5 (the most pertinent).

Afterward, the data were converted into numeric codes, to make the exploitation simplified, as much as the exportation and analysis.

FIELD AND LABORATORY DATA ANALYSIS

Descriptive study. Flock and total aMPV seroprevalence. A flock was considered positive when the geometric mean antibodies titer exceeds 195, corresponding to the CIVTEST AVI TRT® cut-off. Then flock seroprevalence was calculated.

Besides, the percentage of flocks seropositive to aMPV was also calculated along with total seroprevalence. Seroprevalences were also investigated by area and by season.

Statistical analysis. By considering clinical respiratory signs, whether they exist or not, and the seropositivity, flocks can be studied into different groups based on those mentioned above "categorial" variables.

In that case, since the Chi-square test of independence is a statistical hypothesis test used to determine whether two categorical or nominal variables are likely to be related (20), it could demonstrate if an association between seropositivity and respiratory signs exists or not, so, groups of sick birds and healthy birds were compared according to the presence of antibodies.

Also, Multivariate logistic regression models were applied to study the effect of bioclimatic area, season, flock density, single-age or multi-age, ventilation, litter quality, hygiene and biosecurity score, and presence of wild birds as variables (independent variables) on the aMPV serological status (dependant variable) and identify those being eventual risk factors of the aMPV seropositivity after calculating both χ^2 and odd-ratio values.

All the statistical parameters, including the chi-square tests, were calculated with the software IBM SPSS® Statistics 22 (SPSS Inc., Chicago, IL, USA), and the level of significance was stated at $p<0.05$.

RESULTS

Descriptive study. Flock and total aMPV seroprevalence. From the forty-eight flocks, only two showed a mean antibodies titre less than 195. As forty-six flocks showed seropositivity to aMPV as abovementioned, the percentage of herds with deduced aMPV circulation represents 95.83%.

Also, from the 1142 samples analysed, a total of 912 were positive to aMPV with the ELISA test, which corresponds to a seroprevalence of 79.86% with an interval of confidence of 95% [77.41% – 82.15%].

In all the bioclimatic zones, seroprevalence observed was quite high. The Table 2 summarizes the percentages obtained by area and season.

During winter, the highest seroprevalence was observed in the arid bioclimate area (area 3), with

94.16% [89.20% – 97.29%], while a minimum of 62.46% [56.64% – 68.02%], still during the same season, was in the sub-arid zone (area 2).

Analytical study

Association between aMPV seropositivity and presence of clinical signs. Eleven flocks showed various respiratory signs before or during the sampling, including sneezing, coughing, or dyspnoea, unlikely referring to aMPV infection.

Even though, among these farms, one (1-TS59) has shown typical signs of SHS during the visit, including loud coughing and extended head and swollen sinuses.

The test showed a statistically significant association between respiratory signs and seropositivity, with a chi-square value of 14.087 and p-value <0.005.

Statistical analysis of questionnaires data and comparison with serology. Bioclimate area, season, flock density, as well as hygiene and biosecurity score were identified as risk factors for the aMPV seroprevalence, with asymptotic signification (Sig) of the relation between the variable and seropositivity lower than 0.05, Sig<0.05.

Following these findings, logistic regression models were established for every variable to define the effect of each factor on seropositivity, with outcome reported in the Table 3.

Based on these findings, it was concluded that the area 3 (arid zone) presented 55% more risk of aMPV seropositivity than area 1 (humid zone) and 65% more risk than area 2 (sub-humid zone).

Also, the flocks sampled in winter showed 26% less risk of infection than in spring, and the higher densities are predisposing to aMPV infection. In fact, the flocks with > 14 birds/m² tended to present 67% more risk of seropositivity than those reared with densities ranges ≤ 11 birds/m².

Moreover, the farms with poor levels of hygiene (score 1) were three times more susceptible to being infected than farms with good biosecurity levels (risk of 300%).

DISCUSSION

With the high seroprevalence of 79.86% [77.41% – 82.15%] found, it is possible to consider the avian metapneumvorius as an endemic virus, circulating in broiler farms regardless of the bioclimatic area or the season.

Similar seroprevalence was found in Bangladesh (21) and South Korea (22), respectively, with 72.30% and 73.10%.

In the Middle East, avian metapneumovirus serological investigations reported a relatively low prevalence of 21.7% in Egypt (23) and 21.7% of broilers flocks among 47.7% poultry farms in Jordan (24). It should be pointed out that the slaughtering age in these countries is usually around thirty days, quite earlier compared to Morocco, which may explain the results obtained. In fact, the aMPV tends to affect flocks older than 26 days of age (25 – 27), and ELISA antibodies are not detectable before seven days post-infection (28).

It would be highly interesting to know the regional context of the avian metapneumovirus in North Africa. However, the seroprevalence in the surrounding countries is still unknown, unfortunately. In the case of Algeria, the only study regarding the aMPV highlights the circulation of the subtype-B in turkeys flocks (29).

It should be pointed out that resources allocated to the present study allowed carrying the serology tests with one kit only, which is the CIVTEST AVI TRT® (HIPRA S.A., Amer, Spain).

Thus, we encourage future studies using other kits to compare our results because variable results can be obtained with variable specificity, depending on the antigen used and the geographical origin of the virus coated (30).

Considering that all the flocks of the study were strictly not vaccinated against avian metapneumovirus and were at least 35 days of age, it was obvious that the titers detected by ELISA referred to an immune response following a field virus exposure, rejecting any hypothesis of vaccine antibodies or maternal driven ones, the latter supposed to be at their lowest level at two weeks of age (31).

These results align with a previous study confirming the association between seropositivity and clinical respiratory signs, also emphasizing the swollen head syndrome (32).

Almost all the flocks tested showed positive serological results, with 46 positives over 48, but only eleven showed respiratory signs. Thus, it is important to look cautiously at the possible role of aMPV in these symptoms, despite the association between respiratory problems and seropositivity demonstrated by the statistical analysis.

In this regard, studies have proven synergistic effect between aMPV and *E. coli* in co-infections, even without SHS manifestation (33). Besides, it was described that some external factors might contribute to the prevalence of aMPV-infection like density, farm management, ventilation, general hygiene conditions, and veterinary monitoring (34). This evidence supports the suggestion that aMPV infection may occur without SHS symptoms (5), justifying the positivities found in the thirty-seven farms without clinical signs.

Furthermore, the sampling period and the waning of respiratory problems are important to consider and

could explain the absence of symptomatology in 76.86% of the birds that tested positive. In fact, the aMPV shows an incubation period of 2 to 3 days following contact with birds before the wane of respiratory signs and lasts 2 to 3 weeks in the absence of bacterial complications (34). However, seroconversion is detectable by ELISA between 13- and 28-days post-infection (35), which coincides with the period of disappearing clinical signs.

In contrast, respiratory distress has been described in flocks infected exclusively with the avian metapneumovirus (36), with 68.80% of the birds expressing respiratory signs showing positivity to the aMPV when tested by RT-PCR and rejecting for the first time the common belief that avian metapneumovirus is a minor pathogen in broilers.
Farms within the arid bioclimate zone seem to be at high risk of aMPV infection than humid or sub-arid areas. The highest seroprevalence was noticed during winter (94.16%) in this regard.

Emphasizing the arid zone, a wide temperature range dominates the area, varying from warm weather along the Atlantic coast to very cold in the Middle and Anti- Atlas Mountain chains edges (37).

The broiler production is sensitive to temperature variation, proving that heat adversely impacts performance (38). At the same time, cold stress may increase the susceptibility of birds to many infectious pathogens (39). In the case of aMPV, cold weather is likely to preserve the virus, when the stress-induced chickens would lower their immunity (40).

Seropositivity was higher in spring than winter, and surprisingly, our results contradict many previous studies from different parts of the world that reported such findings during the winter (41 – 44).

In this respect, it is important to highlight particularities of the weather and seasons in Morocco, since temperatures may vary during the year according to seasons (45). During winter, the difference between the lowest and highest temperature is less than 5°C, while in spring, it reaches up to 11°C of difference.

One other aspect to consider is the post-COVID19 economic impact. The crisis forced many farmers to stop the activity temporarily and even definitively for some others, with subsequently up to 60% drop of placements until the beginning of 2021 (ONSSA, 2021)—in other words, having many farms empty with few chicks reared would be one of the causes of the lowest prevalence observed in fall, by slowing down the virus transmission from one site to another.

The possible role of the season as a risk factor should be investigated by extending the present study to other seasons, though.

The risk of infection with avian metapneumovirus is also associated with high densities, static ventilation, and poor hygiene. These results follow a previous investigation in Saudi Arabia (32).

It is important to ensure a proper density stock for optimal results. In contrast, negative effects on growth performance and depreciation of welfare conditions in broilers seem to be observed in broilers reared with a density bigger than ten birds/m² (46).

Because it is known for creating promiscuity between birds, farmers seek the crowding to reduce fixed charges and optimize heating costs during cold seasons. Still, this practice is favorable for virus transmission. In this regard, it was demonstrated that the broiler tracheal barrier efficacy would decrease with increasing stocking density (47), justifying the high contaminations.

Although litter quality was not concluded to be a risk factor in the present study, it remains highly impacted by stocking density, to the best of our knowledge. The over-density raises the temperature of the litter, elevating harmful contents such as moisture, dust, or ammonia and leading to respiratory problems (48). This situation is further complicated when ventilation is poor and cannot maintain air quality and adequate relative humidity. Unfortunately, most of the broiler farms in the country rely on static ventilation with curtain-side houses systems. Thus, the litter quality needs more investigation, emphasizing the possible interrelation with density and ventilation.

Our results align those reported elsewhere (32, 49 – 51), since general hygiene, and particularly good biosecurity, help to decrease the load of environmental microorganisms that cause complications, together with best farm management procedures, or the ventilation and proper stock density (1, 52, 53), as previously highlighted.

Besides, a success story in Colorado, United States, showed the possibility of eradicating the subtype-C avian metapneumovirus thanks to biosecurity, alongside other stringent sanitary measures (54).

The human factor and the subjective perception of hygiene are limiting factors toward effective biosecurity. The lack of understanding of biosecurity principles leads to poor consistent application and compliance measures (55).

Likewise, in Morocco, a high level of monitoring is accorded to breeders and layers farms, whereas broilers exploitations with high capacities suffer from under-skilled human resources (17).

Thus, improving observation abilities and skills in management by training is crucial to improve hygiene and, subsequently, disease prevention (56).

The present study states that bioclimate, season, density, and hygiene are the only risk factors associated with aMPV seropositivity. But other

parameters can be of high risk of transmission of the virus and need extended studies in the future.

Interestingly, while collecting field data among veterinarians and farmers during sampling visits, we realized that several farms used to integrate avian metapneumovirus vaccination in their preventative programs in different ways: seasonal during cold seasons or periods of high challenge, for three to four consecutive flocks until stopping incidence of clinical signs, or alternately. Notwithstanding, all of them ceased long ago, either because the advantages of vaccination are not well perceived or to reduce production costs. It is a common belief that the cost/benefit impact of SHS vaccination in broilers is usually underestimated (57).

In the absence of a specific control strategy against avian metapneumovirus in broilers, the virus is likely to contaminate birds and spread among farms, and contaminate successive flocks within the same site. In this regard, vaccination is crucial in the protection against aMPV, particularly in endemic areas with high field virus pressure or co-existing factors in the area (6). It has been proved that considering the vaccination in broilers would lower the circulation of aMPV in many countries, where the infection is reported a lot (4).

Because the number of broilers vaccinated in Morocco is non-significant compared to the total national production, it was not possible in the present study to consider the effect of vaccination of previous flocks to make any definitive conclusion.

The rapid growth of the avian industry in Morocco, particularly broiler chickens, has led to dense poultry regions. Besides, Morocco is known for the very important production of meat turkey, with more than 12.5 million turkey poults placed in 2020 (15). The farms in proximity, especially turkeys, can be exposed to the virus (58), possibly enhancing the virus's spread and dissemination in the environment.

For instance, Souss-Massa and Draa (formerly one cluster Souss-Massa-Draa) represent an important geographic area of broiler production in Morocco, with 1,015 farms authorized between both territories (ONSSA, 2021), corresponding to 12.70% of total farms. It also means an important part of the present study, with 27% of the farms (13 from 48).

Another region, Rabat-Salé-Kénitra, is also as important as the abovementioned, representing 10.24% of total national broiler farms (ONSSA, 2021). Then, we tried to investigate the possible link between close distance to surrounding farms and the aMPV seropositivity. In this case, we analyzed the location of some farms with an aerial view using spatial coordinates.

Farm 1-TS59, which has shown the typical clinical form of SHS aforementioned, is located in the respective region and is close to one commercial layer farm and four other poultry farms.

It should be clarified that several participants in the study were reluctant to share the spatial location of farms for different reasons. Since this method was the only one possible whereby the required identification of surrounding sites and how close they are could be obtained, we couldn't further explore the potential risk associated with geographic location and proximity to other poultry sites.

A possible factor that shouldn't be lessened is the presence of traditional poultry farming in Morocco. With a stable production of 50,000 tons of meat, it is considered an important alternative for increasing animal protein contribution in rural environments. However, diseases and lack of supervision with a median of 10% mortality rates are major constraints of the traditional avian sector (59). Thus, this vulnerable segment could present a source of constant transmission of the virus to high-capacity poultry farms.

The aMPV transmission driven by wild birds has also been hypothesized to the best of our knowledge, at least, for the subtype-C. Whether with a limited replication of aMPV or a partial susceptibility to the infection, migratory birds might play the role of natural reservoirs of infection or mechanical vectors (60 – 62).

With an emphasis on the birds overflying Euro-Mediterranean countries playing a major role in the aMPV-B spread in the last years like Spain, France or Italy (4), or long flyways by extension to Russia and other European countries where, interestingly, aMPV has been reported with a high-frequency detection even in chickens (63 – 66), it seems that indeed the wild birds might play a key role in spreading the virus over long distances.

Thereafter, studies in transitional destinations like Morocco, an important checkpoint of migratory birds' sea crossing (67), become interesting to investigate such a hypothesis. The only available but unpublished data is related to yellow-legged gull *Larus michahellis* in the region of Essaouira. Still, only low pathogenic avian influenza H9N2 and Newcastle disease viruses were studied.

Here again, and due to the very limited information, we couldn't confirm any suggestion between the high seroprevalence found and parameters (that could be) linked to the presence of traditional farms or wild birds.

CONCLUSIONS

Despite that avian metapneumovirus is a contagious virus, the high seroprevalence obtained

was quite unexpected regardless of the region, particularly in farms without clinical signs.

Bioclimate, season, density, and general hygiene contribute as risk factors to the virus circulation and, at their utmost, to respiratory problems.

Particular attention should also be given to possible consequences of the aMPV circulation which are not "perceived."

As a first example, the clinical signs and laboratory investigations during acute respiratory syndromes, in the absence of head swelling, often focus on avian influenza or infectious bronchitis, as described previously. In addition to that, the monitoring of body weight in most conventional broiler flocks in Morocco is based on visual observation only. Therefore, the appreciation of any eventual lowered daily gain is difficult, and, subsequently, any possible impact of AMPV-infection on the bodyweight is also underestimated. Furthermore, because more than 90% of the national broiler chicken production is intended for the live market, evaluating the yield condemnations and depreciation that could be associated with avian metapneumovirus lesions is also not possible.

It is clear then that the aMPV is highly circulating among farms in Morocco, likewise many other countries. While the reasons behind this scenario remain not fully clear, though, and available data are still poor, more research is needed to understand the virus's behavior in the field.

Accordingly, studies should focus on season extension, identifying possible other risk factors by increasing samples over time. The use of other available commercial ELISA kits can be relevant to compare the present results and improve their accuracy.

Besides, since the molecular tools offer rapid and reliable results with good sensitivity and specificity today, upcoming investigations must emphasize detecting the avian metapneumovirus using RT-PCR. The results obtained would aim to identify the circulating subtype(s), and sequencing perspectives, coupled with our serological evidence, would help understand more epidemiological aspects, particularly the avian metapneumovirus.

(The full-length article will be published in a journal to be determined later.)

ACKNOWLEDGMENT

We thank all the veterinarians and producers whether for giving us access to farms and pieces of information or for providing us with sera samples.

REFERENCES

1. Hafez, H. The role of pneumovirus in swollen head syndrome of chickens: review. *Arch Fur Geflugelkd* ;57(4):181-185.

2. Gough, R.E.; Manvell, R.J.; Drury, S.E. and Pearson, D.B. 1994. Isolation of an avian pneumovirus from broiler chickens. Vet Rec, 134, 353-354. 1993.

3. Jones, R.C. Avian pneumovirus infection: questions still unanswered. Avian Pathol, 25, 639-648. 1996.

4. Franzo, G.; Legnardi, M.; Mescolini, G.; Tucciarone, C.M.; Lupini, C.; Quaglia, G.; Catelli, E. and Cecchinato, M. Avian Metapneumovirus subtype B around Europe: a phylodynamic reconstruction. Vet Res. 51(1):1-10. 2020.

5. Owoade, A.A.; Ducatez, M.F. and Muller, C. P. Seroprevalence of avian influenza virus, infectious bronchitis virus, reovirus, avian pneumovirus, infectious laryngotracheitis virus, and avian leukosis virus in Nigerian poultry. Avian Disease. 50, 222-227. 2006.

6. Tucciarone, C.M.; Franzo, G.; Lupini, C.; Alejo, C.T.; Listorti, V.; Mescolini, G.; Brandao, P.E.; Martini, M.; Catelli, E. and Cecchinato, M. Avian Metapneumovirus circulation in Italian broiler farms Poult. Sci., 97, pp. 503-509. 2018.

7. Cook, J.K.A and Cavanagh, D. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathol; 31 (2):117-132. 2002.

8. Banet-Noach, C.; Simanov, L. and Perk, S. Characterization of Israeli avian metapneumovirus strains in turkeys and chickens. Avian Pathol. 34:220-226. 2005.

9. Jones, R.C. Viral respiratory diseases (ILT, aMPV infections, IB): Are they ever under control? Brit. Poult. Sci. 51(1):1-11. (Review). 2010.

10. Dos Santos, M.B.; Martini, M.C.; Ferreira, H.L.; Da Silva, H.A.L.; Fellipe, P.A.; Spilki, F.R. and Arns, C.W. Brazilian avian metapneumovirus subtypes A and B: Experimental infection of broilers and evaluation of vaccine efficacy. Pesqui Vet Bras.;32(12):1257-1262. 2012.

11. Arthanari Eswaran, M.; Sukumar, K.; Johnson Rajeswar, J.; Balasubramaniam, G.A. and Anna, T. Serological Survey of Avian Metapneumovirus Infection in Broiler Breeder Chicken Farms in Tamil Nadu. Int.J.Adv.Vet.Sci.Tech., 3(1):84-87. 2014.

12. Cook, J. K. A. Avian pneumovirus Infections of Turkeys and Chickens. Vet.J., 160: 118-125. 2000.

13. Ruthra, M.; Arulmozhi, A.; Balasubramaniam, A.; Balasubramaniam, G.A. and

Madheswaran, R. Seroprevalence of avian Metapneumovirus (Ampv) in layer flocks in namakkal. Indian Vet J. 94(10):13-15. 2017.

14. Collins, M.S.; Gough, R.E. and Alexander, D.J. Antigenic differentiation of avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. Avian Pathol 22:469–479. 1993.

15. Fédération Interprofessionnelle du Secteur Avicole (FISA). Production reports.

http://www.fisamaroc.org.ma/index.php?option=com_content&view=article&id=65&Itemid=49.

Accessed on Sept 2, 2021.

16. Mokhtari, N.; Mrabet, R.; Lebailly, P. and Bock, L. Spatialisation des bioclimats, de l'aridité et des étages de végétation du Maroc. Revue Marocaine des Sciences Agronomiques et Vétérinaires 2, p.50-66. 2013.

17. Barkok, A. Structure et importance des secteurs avicoles commercial et traditionnel au Maroc. Revue du Secteur Avicole au Maroc, FAO pp: 3 – 10. 2007.

18. El Houadfi, M.; Hamam, A.; Vanmarcke, J. and Cook, J.K.A. Swollen head syndrome in broiler chickens in Morocco. *In* Proc. 40th Western Poultry Disease Conference, 24-27 April, Acapulco. University of California, Davis, 126-127. 1991.

19. Pfeiffer, D.U. Sampling of Animal Populations. In: Veterinary Epidemiology – An Introduction. Pfeiffer, D.U. The Royal Veterinary College, University of London. pp: 34-36. 2002.

20. Statistics Knowledge Portal: a Free Online Introduction to Statistics. Chi-Square Test of Independence.

https://www.jmp.com/en_ch/statistics-knowledge-portal/chi-square-test/chi-square-test-of-independence.html

Accessed on Jan 15, 2022.

21. Ali, M.Z.; Park, J.E.; and Shin, H.J. Serological survey of avian metapneumovirus infection in chickens in Bangladesh. J. Appl. Poult. Res. 28, p.1330–1334. 2019.

22. Park, J.E.; Lee, D.W. and Shin H.J. Serological survey of antibodies against avian Metapneumovirus in Korean chicken flocks. J. Appl. Poult. Res. 20, p.573–576. 2011.

23. Nagy, A.; Abdallah, F.; Sweed, K.; Salama, A. and Omar M. Seroprevalence of avian Metapneumovirus in Egyptian chicken and duck flocks with a reference on economic impact. Journal of Virology Science 4, p.8-14. 2018.

24. Gharaibeh, S.M. and Algharaibeh, G.R. Serological and molecular detection of avian pneumovirus in chickens with respiratory disease in Jordan. Poultry Science 86, p.1677–1681. 2007.

25. Giraud, P. and Bennejean, G. Turkey rhinotracheitis in France: preliminary investigations on ciliostatic virus. Vet. Rec. 119, p.606-607. 1986.

26. Aung, Y. H.; Liman, M.; Neumann, U. and Rautenschlein, S. Reproducibility of swollen sinuses in broilers by experimental infection with avian metapneumovirus subtypes A and B of turkey origin and their comparative pathogenesis. Avian Pathol 37, p.65-74. 2008.

27. Franzo, G.; Legnardi, M.; Mescolini, G.; Tucciarone, C.M.; Lupini, C.; Quaglia, G.; Catelli, E. and Cecchinato, M. Avian Metapneumovirus subtype B around Europe: a phylodynamic reconstitution. Vet Res 51, p.88. 2020.

28. Jirjis, F.F.; Noll, S.L.; Halvorson, D.A.; Nagaraja, K.V. and Shaw, U.D.P. Pathogenesis of avian pneumovirus infection in turkeys. Vet. Pathol. 39, 300-310. 2002.

29. Sid, H.; Benachour, K. and Rautenschlein, S. Co-infection with multiple respiratory pathogens contributes to increased mortality rates in Algerian poultry flocks. Avian Diseases 59, p.440-446. 2015.

30. Eterradosi, N.; Toquin, D.; Guittet, M. and Bennejean, G. Discrepances in Turkey Rhinotracheitis ELISA results using different antigens. Veterinary Record 131: 563-564. 1992.

31. Rubbenstroth, D. and Rautenschlein, S. Investigations on the protective role of passively transferred antibodies against avian Metapneumovirus infection in turkeys. Avian Pathol 38, p.427–436. 2009.

32. Al-Ankari, A.R.S.; Al-Ramadan, M.A. and El-Demerdash, M.M. Risk Factors Associated with Prevalence of Swollen Head Syndrome (SHS) in Broiler Chickens in Eastern Province – Saudi Arabia. International Journal of Poultry Science 3, p-646-650. 2004.

33. Al-Ankari, A.R.S.; Bradbury, J.M.; Naylor, C.J.; Worthington, K.J.; Payne-Johnson, C. and Jones, R.C. Avian pneumovirus infection in broiler chicks inoculated with Escherichia coli at different time intervals. Avian Pathol. 30, p.257–267. 2001.

34. Jones, R.C.; Baxter-Jones C.; Savage, C.E.; Kelly, D.F. and Wilding, G.P. Experimental infection of chickens with a ciliostatic agent isolated from turkeys with rhinotracheitis. The veterinary record. 120, p.301-320. 1987.

35. Aung, Y.H.; Liman, M.; Neumann, U. and Rautenschlein, S. Reproducibility of swollen sinuses in broilers by experimental infection with avian metapneumovirus subtypes A and B of turkey origin and their comparative pathogenesis. Avian Pathol. 2008; 37(1):65-74. 2008.

36. Tucciarone, C.M.; Franzo, G.; Lupini, C.; Alejo, C.T.; Listorti, V.; Mescolini, G.; Brandao, P.E.; Martini, M.; Catelli, E. and Cecchinato, M. Avian

Metapneumovirus circulation in Italian broiler farms Poult. Sci., 97 (2018), pp. 503-509. 2018.

37. Mokhtari, N.; Mrabet, R.; Lebailly, P. and Bock, L. Spatialisation des bioclimats, de l'aridité et des étages de végétation du Maroc. Revue Marocaine des Sciences Agronomiques et Vétérinaires 2, p.50-66. 2013.

38. Tawfeek, S.S.; Hassanin, K.M.A. and Youssef, I.M.I. The effect of dietary supplementation of some antioxidants on performance, oxidative stress, and blood parameters in broilers under natural summer conditions. Journal of World's Poultry Research, 4(1), 10 – 19. 2014.

39. Huff, G.; Huff, W.; Rath, N.; Solis de los Santos, F.; Farnell, B. and Donoghue, A. Influence of hen age on the response of turkey poults to cold stress, Escherichia coli challenge, and treatment with a yeast extract antibiotic alternative. Poultry Science, 86, 636–642. 2007.

40. Bakre, A.A.; Oladele, O.A.; Oluwayelu, D.O. and Esan, O.O. Serological survey for avian Metapneumovirus in commercial chickens from different climatic zones in Nigeria. J Vet Med Anim Heal.;12(3):110-115. 2020.

41. Tanaka, M.; Takuma, H.; Kokumai, N.; Oishi, E.; Obi, T.; Hiramatsu, K.; and Shimizu, Y. Turkey rhinotracheitis virus isolated from broiler chicken with swollen head syndrome in Japan J. Vet. Med. Sci., 57 (1995), pp. 939-941. 1995.

42. Kwon, J.S; Lee, H.J.; Jeong, S.H.; Park, J.Y.; Hong, Y.H.; Lee, Y.J.; Youn, H.S.; Lee, D.W.; Do, S.H.; Park, S.Y.; Choi, I.S.; Lee, J.B. and Song, C.S. Isolation and characterization of avian Metapneumovirus from chickens in Korea J. Vet. Sci., 11, pp. 59-66. 2010.

43. Seifi, S. and Boroomand, Z. The Role of Avian Metapneumovirus in Respiratory Complex Disease Circulating in Broilers in Northern Iran. Trakia J Sci. 2015; 13(2):175-179. 2015.

44. Ali, M.Z.; Park, J.E. and Shin, H.J. Serological Survey of Avian Metapneumovirus Infection in Chickens in Bangladesh. J Appl Poult Res. 2019;28(4):1330-1334. 2019.

45. Climate Change Knowledge Portal. The current climate, climatology of Morocco. https://climateknowledgeportal.worldbank.org/country/morocco/climate-data-historical. Accessed on September 4th, 2021.

46. Cai, C.H.; Zhao, Wang, P.; Wang, J.S.; Li, K.X.; Zhan, X.A. and Wang, K.Y. Effects of different stocking densities on growth performance, antioxidant ability, and immunity of finishing broilers. Anim. Sci. J. 90:583–588. 2019.

47. Wang, Y.; Wang, D.; Wang, J.; Li, K.; Heng, C.; Jiang, L.; Cai, C. and Zhan, X. Effects of different stocking densities on tracheal barrier function and its metabolic changes in finishing broilers. Poultry Science 99: 6307–6316. 2020.

48. Meluzzi, A. and Sirri, F. Welfare of broiler chickens. Ital. J. Anim. Sci. 8:161–173. 2009.

49. Goodwin, M.A. and Waltman, W.D. Clinical and pathological findings in young Georgia broiler chickens with oculo-facial respiratory diseases "so called swollen head". Avian Dis., 3: 376-378. 1994.

50. Morely, A.G. and Thomson, D.K. Swollen head syndrome in broiler chickens. Avian Dis., 28: 238- 243. 1984.

51. Naylor, C.J. and Jones, R.C. Turkey rhinotracheitis: a review. Vet. Bul., 63: 439-449. 1993.

52. Pagès, A and San Gabriel, A. Consideraciones actuales del Síndrome de cabeza hinchada en las aves. Medicina Veterinaria 7: 243-250. 1990.

53. Shane, S.M. The Swollen Head Syndrome: an emerging condition in chickens. Vineland update 38. 1991.

54. Suarez, D. L., Miller, P.J., Koch, G., Mundt, E. and S. Rautenschlein, Newcastle Disease, Other Avian Paramyxoviruses, and Avian Metapneumovirus Infections. In: Diseases of Poultry 14th Edition, David E. Swayne. pp: 109 – 166. https://doi.org/10.1002/9781119371199.ch3. 2019.

55. Racicot, M. and Vaillancourt, J.P. Evaluation of biosecurity measures based on video surveillance in poultry farms in Quebec and main failures. Bulletin de l'Académie vétérinaire de France 162(3) pp: 265 – 272. 2009.

56. Smith, J.A.; Boulianna, M.; Owen, R.L. and Gingerich, E. Disease Prevention and Control in Antibiotic-Free Production. In: Swayne DE. Diseases of Poultry 14th Edition pp: 40 – 51. 2020.

57. Bayraktar, E.; Umar, S.; Yilmaz, A.; Turan, N.; Franzo, G.; Tucciarone, C.M.; Cecchinato, M.; Cakan, B.; Iqbal, M. and Yilmaz, H. First molecular characterisation of avian Metapneumovirus (aMPV) in Turkish broiler flocks. Avian Dis. 2018 Dec 1; 62(4):425-430. 2018.

58. Lupini, C.; Cecchinato, M.; Ricchizzi, E.; Naylor C.J. and Catelli, E. A turkey rhinotracheitis outbreak caused by the environmental spread of a vaccine-derived avian metapneumovirus. Avian Pathol 40, p.525-530. 2011.

59. Chahbi, A.; El Hadrami, E.; El Amri, H.; Nassik, S.; Douaik, A. and Boukharta, M. Non-industrial poultry farming in Morocco: A survey study in the Rabat-Salé-Kénitra region. Rev. Mar. Sci. Agron. Vét. 7(3): 437-446. 2019.

60. Turpin, E.A.; Stallknecht, D.E.; Slemons, R.D.; Zsak, L and Swayne, D.E. Evidence of avian metapneumovirus subtype C infection of wild birds in

Georgia, South Carolina, Arkansas and Ohio, USA. Avian Pathology, 37:3, 343-351. 2008.

61. Gharaibeh, S. and Shamoun, M. Avian Metapneumovirus Subtype B Experimental Infection and Tissue Distribution in Chickens, Sparrows, and Pigeons. Vet Pathol. 2012; 49(4):704-709. 2012.

62. Rizotto, L.S.; Simão, R.M.; Scagion, G.P.; Simasaki, A.A.; Caserta, L.C.; Benassi, J.C.; Arns, C.W. and Ferreira, H.L. Detection of avian metapneumovirus subtype A from wild birds in the state of São Paulo, Brazil. Pesqui Vet Bras. 2019; 39(3):209-213. 2019.

63. Franzo, G.; Tucciarone, C.M.; Enache, M.; Bejan, V.; Ramo, G.; Koutoulis, K.C. and Cecchinato, M. First Report of Avian Metapneumovirus Subtype B Field Strain in a Romanian Broiler Flock during an Outbreak of Respiratory Disease. Avian Dis. 2017; 61(2):250-254. 2017.

64. Andreopoulou, M.; Franzo, G.; Tucciarone, C.M.; Prentza, Z.; Koutoulis, K.C.; Cecchinato, M. and Chaligiannis, I. Molecular epidemiology of infectious bronchitis virus and avian Metapneumovirus in Greece. Poult Sci 98:5374–5384. 2019.

65. Tucciarone, C.M.; Andreopoulou, M.; Franzo, G.; Prentza, Z.; Chaligiannins, I. & Cecchinato, M. First identification and molecular characterization of Avian metapneumovirus subtype B from chickens in Greece. Avian Dis 61:409–413. 2017.

66. Tucciarone, C.M.; Franzo, G.; Lupini, C.; Alejo, C.T.; Listorti, V.; Mescolini, G.; Brandao, P.E.; Martini, M.; Catelli, E. and Cecchinato, M. Avian Metapneumovirus circulation in Italian broiler farms Poult. Sci., 97 (2018), pp. 503-509. 2018.

67. Santos, C.D.; Silva, J.P.; Munoz, A.R.; Onrubia, A. and Wikelski, M. The gateway to Africa: What determines sea crossing performance of a migratory soaring bird at the Strait of Gibraltar? J. Anim. Ecol.89:1317–1328. 2020.

Table 1. Number of flocks selected for investigation according to bioclimatic area.

Bioclimatic area	Identification	Number of farms authorized	Number of flocks to investigate	
			Winter	Spring
Humid & sub-humid	Area 1	1945	5	5
Sub-arid	Area 2	4200	11	11
Arid	Area 3	1710	8	8
Hyper-arid*	Area 4	137	0	0
Total		7992	24	24

(*) For logistic issues, we couldn't collect samples from the hyper-arid area.

Table 2. Seroprevalence according to bioclimatic area.

Area	Winter				Spring			
	Sera	**(+)**	**SP* (%)**	**GMT****	**Sera**	**(+)**	**SP* (%)**	**GMT****
1	128	116	90.63 [84.20 – 95.06]	519.16 ± 124.30	130	88	67.69 [58.93 – 75.62]	315.25 ± 186.25
2	293	183	62.46 [56.64 – 68.02]	364.28 ± 348.06	246	218	88.62 [83.97 – 92.30]	867.21 ± 742.90
3	154	145	94.16 [89.20 – 97.29]	1043.58 ± 665.82	191	162	84.82 [78.93 – 89.59]	662.18 ± 301.94
Total	575	444	77.22 [73.57 – 80.58]	622.98 ± 300.63	567	468	82.54 [79.16 – 85.58]	697.62 ± 570.09

Sera = total number of sera analysed; (+) = positive sera; SP (%) = seroprevalence; GMT = Geometric mean antibodies titre

*SP are indicated with confidence intervals of 95%

**GMT are indicated with the standard deviation

Table 3. Binary logistic regression model of variables associated to aMPV seroprevalence in broiler farms.

Factors	Exp(B)	CI 95%	Sig
Bioclimatic area			
Humid and sub-humid	0.45	0.28 – 0.71	0.000*
Sub-arid	0.35	0.23 – 0.52	0.001*
arid	Ref		
Season			
Winter	0.74	0.55 – 0.99	0.049*
Spring	Ref		
Density			
≤ 11 birds/m²	0.33	0.22 – 0.51	0.000*
11 – 14 birds/m²	0.74	0.49 – 1.14	0.172 ^{NS}
> 14 birds/m²	Ref		
Hygiene			
Poor	3.11	1.73 – 5.58	0.000*
Average	1.21	0.86 – 1.72	0.277 ^{NS}
Good	Ref		

*Significant value (Sig<0.05); ^{NS} Non-significant value

C.A. = code attributed; N = number; P+ = positives; % = percentage; Sig= significance; Ref = reference and Exp(B) = the chi-square value

AVIAN REOVIRUS SURVEILLANCE IN POULTRY FARMS ON THE UNITED STATES WEST COAST (2012-2029)

P. Montine[A], S. Stoute[B], H.L. Shivaprasad[C], B. Crossley[D], C. Corsiglia[E], and R.A. Gallardo[A]

[A]Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis.
1089 Veterinary Medicine Drive, 4008 VM3B, Davis, CA 95616
[B]California Animal Health and Food Safety Lab, Turlock branch, University of California, Davis.
1550 N Soderquist Rd, Turlock, CA 95380
[C]California Animal Health and Food Safety Lab, Tulare branch, University of California, Davis.
18830 Rd 112, Tulare, CA 93274
[D]California Animal Health and Food Safety Lab, Davis branch, University of California, Davis.
620 Health Science Dr, Davis, CA 95616
[E] Foster Farms, Livingston, CA, 95334

SUMMARY

Avian reovirus (ARV) which causes viral arthritis-tenosynovitis, immunosuppression, and lameness leads to condemnation of birds and subsequent economic loss. This segmented virus is subject to variability due to mutations and recombination events reason why requires a constant active surveillance approach to better understand its behavior and plan prevention and control strategies. Even though ARV surveillance in broiler chickens has been performed for years, we are not aware of studies performed to determine patterns or trends of ARV detection over time. The goal of this project is to understand ARV seasonality, determine the predominant variants over the years and understand the spatial and temporal distribution of cases. Diagnostic laboratory reports from a single broiler company were compiled and analyzed. Case confirmation was based on positive virus isolation or RT-PCR since diagnostic techniques have varied over the years. The data was analyzed using maps and phylogenetic trees to compare S1 gene sequences.

INTRODUCTION

Avian reovirus (ARV) variants have been reported globally since 2003 (1). In 2010-2011, an outbreak of viral arthritis and tenosynovitis was associated with variant ARV strains in the US. Clinically, the most common problems associated with current ARV variants are viral arthritis-tenosynovitis, pericarditis, and immunodepression, leading to reduced weight gain, lameness, secondary infections, and condemnation (2). ARV belongs to the *Reoviridae* family. Its genome is composed of double-stranded RNA with 10 segments (3). The virus is highly variable, in part due to its segmented nature, which favors mutations, recombination, and reassortment events during viral replication (4). The sigma C protein is important for cell infection and is encoded by the S1 gene. This gene has been associated with antigenicity and is used for reovirus classification and characterization (5) in molecular surveillance programs. ARV variants are constantly emerging, underscoring the importance of epidemiological surveillance in commercial poultry. Surveillance is necessary to detect emerging variants in a geographic location, to identify spatial trends in ARV variability, and to evaluate the currently used autogenous vaccines and biosecurity strategies (5). Currently, surveillance is used to select virus isolates to formulate vaccines to protect against reovirus in the field. The results of surveillance efforts are once again important for stakeholders to address concerns, propose solutions and inform on disease prevention and control measures.

MATERIALS AND METHODS

ARV cases. The study design is a retrospective cross-sectional study looking at the ARV surveillance system of a single broiler chicken company located in California. One submission consists of approximately eight chickens. Tendons, hearts, and hock joint swabs were individually collected, pooled by submission and tested for the presence of ARV by virus isolation or RT-PCR.

Descriptive statistics and mixed effect logistic regression. Descriptive statistics was performed using SAS Studio. Data was input as follows: 1 = Positive reovirus sample pool; 0 = Negative reovirus sample pool. Data generated included genotype, age and number of birds. Seasonality was calculated based on the submission dates. A mixed logistic regression model was made to evaluate if ARV cases were

associated with a particular season. Significance was determined at P<0.05.

Spatial and spatial-temporal analysis. Using the city and county location data, we georeferenced all the commercial poultry farms within the study (n=251). We examined the data for the detection of clusters of positivity (hot spots) or negativity in farms (cold spots) per geographic location and evaluated it using the Bernoulli model and the space-time scan statistic in SaTScan™ version 10.0 (6). Clusters are given a relative risk and a p value which was evaluated using Monte Carlo computer simulation for statical significance (P<0.05).

Temporal phylogenetic analysis. Nextstrain was used to track the evolution of genomic data of ARV sequences (n=67). Metadata was combined with sequencing data. Sequences were aligned and a phylogenetic tree was built. Annotations with inferred ancestor pathogen dates were recorded (7). Trees were built using the maximum likelihood method with 1,000 bootstraps.

RESULTS AND DISCUSSION

ARV cases. A total of 2,079 chickens suspicious of ARV infection were submitted in 251 submission groups (~ 8 chickens per submission) to one of the four CAHFS branches between January 2012 and December 2019. From 351 sample pools collected and tested by virus isolation and RT-PCR 22.51% (79/351) were negative and 77.49% (272/351) were positive. This data demonstrates how prevalent are ARV's in broiler chickens. It is important to discuss that before 2016 the method used for ARV diagnosis was viral isolation which has a very low sensitivity compared with RT-PCR. In addition, RT-PCR strategies have changed with time adding to the sensitivity of the test. Considering this information, these values are underestimated. A subset of 67 positive samples were molecularly characterized through RT-PCR and sequencing demonstrating the presence of genotypic clusters (GC) 1(n=33), 2 (n=8), 3 (n=1), 4 (n=4), 5 (n=1) and 6 (n=20) illustrating the variability of this virus (Table. 1).

Seasonality. Seasonality was not detected when cases were evaluated throughout time of the year, with most cases reported from 2016 to 2019. (Figure 1). This conclusion is supported by the mixed-logistic regression. The model shows that seasons are non-significant (Table 2). These results are not surprising since ARV is a non-enveloped and persistent virus that is vertically transmitted from breeders and horizontally disseminated in poultry flocks.

Spatial and spatial-temporal analysis of ARV. The spatial clustering distribution of ARV samples from 2012-2019 shows a hotspot around the cities of Fresno, Visalia, and Hanford (RR= 1.22, P= 0.331). In contrast a single cold spot was detected around the city of Turlock and Modesto (RR=0.80, P-value= 0.483). Neither were significant. (Fig. 2A)

Meanwhile the spatial temporal clustering of ARV sample pools detected a hotspot (2016-2019) around the city of Fresno, Visalia, and Hanford (RR=1.25, P-value= 0.068). While a cold spot (2016-2019) was detected on the west coast north of Merced (RR= 0.85, P-value= 0.99). Neither were significant. (Fig 2.B). The lack of significance in these clusters suggests that ARV is evenly distributed in poultry flocks. This is not surprising since broiler flocks are generated from the same breeders that might be vertically transmitting this virus to their progeny. In addition, this virus is endemic in poultry populations (8).

Phylogenetic tree. The phylogenetic tree shows the viral evolution of ARV in California. The data used to construct this tree is limited and more sequences are needed in order to increase the power of the phylogeny. Figure 3 shows that GC1 (dark blue) strains were the most prominent from 2015 to 2018. The common ancestors of the depicted ARV variants are believed to be an ARV that potentially existed in 1981. This information supports the variant nature of these strains that share the same precursors (Figure 3).

REFERENCES

1. Kant A, Balk F, Born L, Van Roozelaar D, Heijmans J, Gielkens A, Huurne A Ter. Classification of Dutch and German avian reoviruses by sequencing the σ C protein. Veterinary Research. 2003 [accessed 2021 Nov 6];34(2):203–212. https://hal.archives-ouvertes.fr/hal-00902739. doi:10.1051/vetres:2002067ï

2. Crispo M, Stoute ST, Hauck R, Egaña-Labrin S, Sentíes-Cué CG, Cooper GL, Bickford AA, Corsiglia C, Shivaprasad HL, Crossley B, et al. Partial molecular characterization and pathogenicity study of an avian reovirus causing tenosynovitis in commercial broilers. Avian Diseases. 2019;63(3):452–460. doi:10.1637/12013-121418-Reg.1

3. Benavente J, Martínez-Costas J. Avian reovirus: Structure and biology. Virus Research. 2007;123:105–119. doi:10.1016/j.virusres.2006.09.005

4. De Carli S, Michel Wolf J, Gräf T, M Lehmann FK, K Fonseca AS, Canal CW, Lunge VR, Ikuta N, Gonçalo Moniz I, Oswaldo Cruz F. Genotypic characterization and molecular evolution of avian reovirus in poultry flocks from Brazil. 2020. https://doi.org/10.1080/03079457.2020.1804528. doi:10.1080/03079457.2020.1804528

5. Palomino-Tapia V, Mitevski D, Inglis T, van der Meer F, Abdul-Careem MF. Molecular characterization of emerging avian reovirus variants isolated from viral arthritis cases in Western Canada 2012–2017 based on partial sigma (σ)C gene. Virology. 2018;522:138–146. doi:10.1016/J.VIROL.2018.06.006

6. Kulldorff M, Heffernan R, Hartman J, Assunção R, Mostashari F. A Space–Time Permutation Scan Statistic for Disease Outbreak Detection. PLOS Medicine. 2005 [accessed 2021 Nov 24];2(3):e59. https://journals.plos.org/plosmedicine/article?id=10.1371/journal.pmed.0020059. doi:10.1371/JOURNAL.PMED.0020059

7. Hadfield J, Megill C, Bell SM, Huddleston J, Potter B, Callender C, Sagulenko P, Bedford T, Neher RA. NextStrain: Real-time tracking of pathogen evolution. Bioinformatics. 2018;34(23):4121–4123. doi:10.1093/bioinformatics/bty407

8. Ter Veen C, De Bruijn ND, Dijkman R, De Wit JJ. Avian Pathology Prevalence of histopathological intestinal lesions and enteric pathogens in Dutch commercial broilers with time Prevalence of histopathological intestinal lesions and enteric pathogens in Dutch commercial broilers with time. 2016 [accessed 2022 Feb 3]. https://www.tandfonline.com/action/journalInformation?journalCode=cavp20. doi:10.1080/03079457.2016.1223271

Table 1. Frequency of avian reovirus genotypic clusters diagnosed in broilers on the U.S. west coast from 2012 to 2019.

Genotype	Frequency (n=67)	Percentage
GC 1	33	49
GC 2	8	12
GC 3	1	1
GC 4	4	6
GC 5	1	1
GC 6	20	30

Table 2. Mixed effects logistic regression of association between sample pool diagnosis and seasonality among broilers on the United States west coast from 2012-2019.

Seasons	Odds ratio	95% confidence interval		*P* value
Fall vs spring	0.387	0.001	999.999	0.3866
Winter vs spring	1.87	0.003	127.000	0.3104
Summer vs spring	0.710	0.028	147.660	0.5642

*Significant ($P<0.05$)

Figure 1. Frequency of avian reovirus sample pools consecutively reported in broiler farms on the U.S west coast from 2012-2019 divided by seasons.

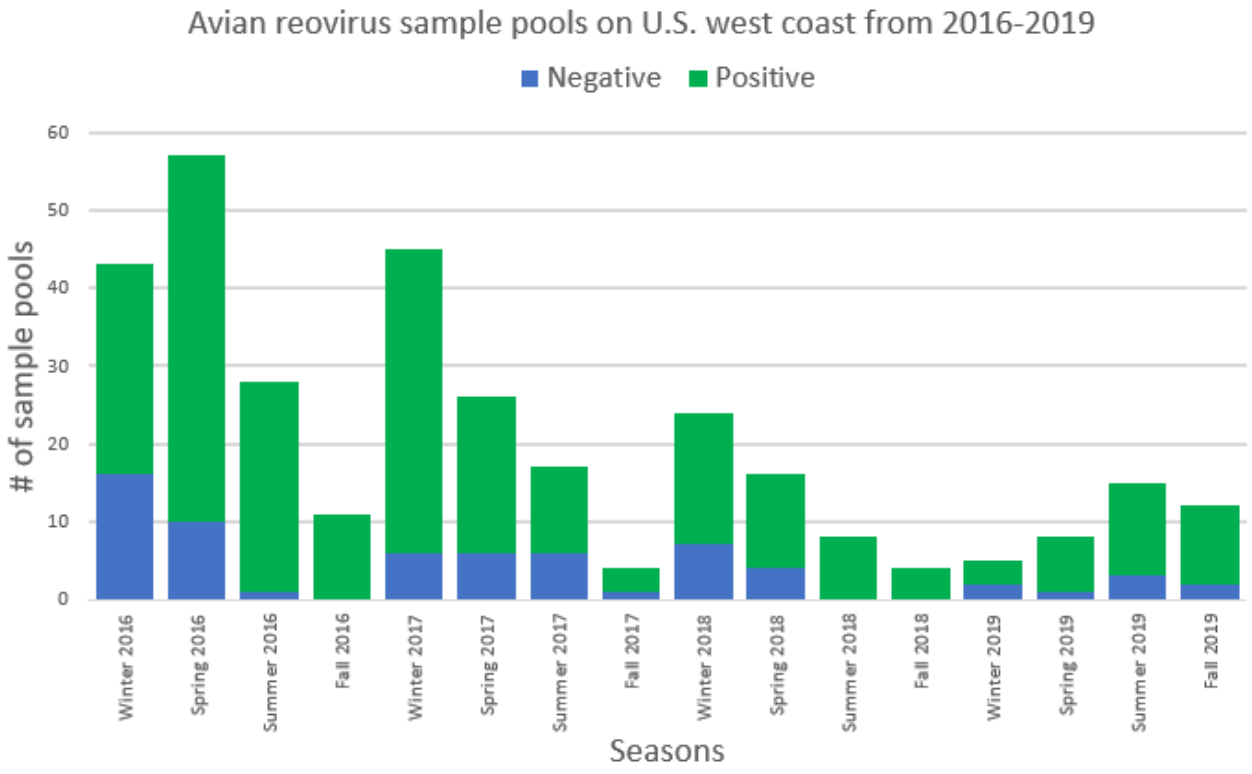


Figure 2. Spatial (A) and spatial-temporal distribution (B) of avian reovirus-positive (hot spots, red) and -negative (cold spots, blue) sample pools by RT-qPCR or Virus Isolation on U.S west coast from 2012-2019.

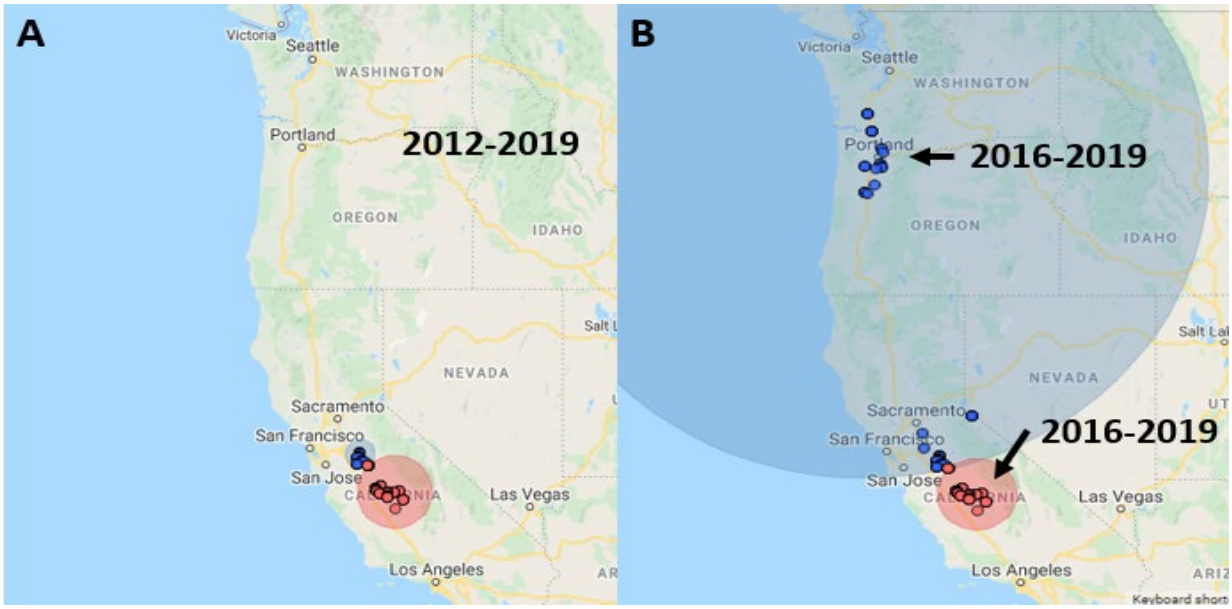
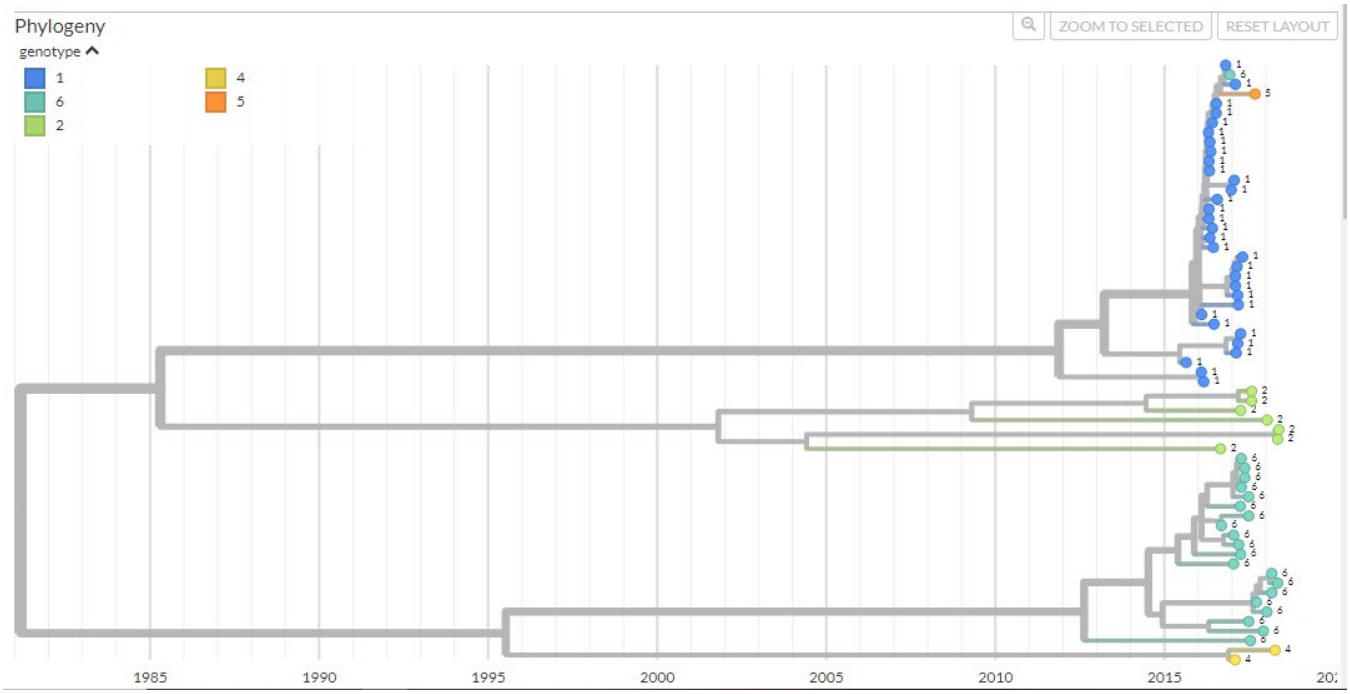


Figure 3. Temporal phylogenetic analysis of avian reovirus strains detected on U.S west coast from 2012-201.



NONINVASIVE PROTOCOL TO ASSESS EGGSHELL HYGIENE STATUS USING LUMINOMETRY

R. Munoz[A], K. Rincon[B], C. Ardila[B], J. Lopez[C], and M. Sanabria[D]

[A]Neogen, Rochdale, UK
[B]Incubacol, Colombia
[C]U. Nacional, Colombia
[D]Ceva Animal Health

SUMMARY

Hygiene and sanitation at the hatchery level have become a critical aspect of poultry production in order to reduce the potential number of contaminants affecting broilers, as well potential antibiotic usage and treatment incidence.

The fertile egg's surface represents a continuous challenge for hatchery biosecurity and the hygiene status of hatchery surfaces. Various factors may affect the hygiene quality of the eggshells of fertile eggs, including nest material, nest management, percentage of eggs laid on target, feces contamination, etc.

An easy-to-use screening type of protocol used to evaluate the hygiene quality of the eggshells of fertile eggs may be valuable in deciding when to intervene in those eggshells and whether those interventions are successful or if a different type of intervention, or no further action, is required.

INTRODUCTION

The use of luminometer technology in the food safety industry has been proof of the great value the screening sanitation tool provides, with timely results and easy-to-use data that assesses critical points on hard inert surfaces[1]. The 10 cm x 10 cm (100 square centimeters) surface sampled area has also provided proof of consistency within other parameters of hygiene indexes in food safety surfaces environments and conditions, keeping a more consistent reproducibility of the protocol, resulting in lower possible variability at sampling technique[2]. These 100 square centimeters have been used in other sanitation studies, such as aluminum-based surfaces in swine transport[3] and other material-based surfaces in poultry hatchery studies[4]. In those studies, the expected trend of relative luminescent units (RLU) was shown and had an acceptable correlation with other methodologies. Under experimental conditions, the sampling of eggshells with luminometers and simultaneous bacteria count has shown acceptable levels of correlation[5].

OBJECTIVE

To obtain potential eggshell residual contamination data through indirect monitoring using plastic food wrap-up, hard surface swab samplers, and an AccuPoint® Advanced reader.

MATERIALS AND METHODS

Materials:

Eggs tray to be sampled
Empty egg tray
Adhesive food wrap-up plastic roll
Scissors
Gloves
Push pins
AccuPoint reader
Surface samplers

Method. Draw a template and bookmark on the table surface (10 cm x 10 cm).

Put on gloves before handling. Bring the egg tray to be sampled.

Place and cover the egg tray using the adhesive food plastic with the sticky side on top of the 30 eggs tray; it must be the internal face, leaving a margin of around10 cm between each end (borders). See Figure 1.

Cover the food wrap-up plastic with the empty egg tray to make sure most eggshell surfaces could get into contact with the sticky side of the food wrap-up plastic and, using the scissors, cut the end of the remaining wrap-up plastic. See Figure 2.

When removing it, make sure that the food wrap-up plastic that had been in contact with the eggshells does not have contact with any other surface. Now place it on the 10 cm x 10 cm reference area that you have drawn, and fix the four corners using the four push pins. See Figure 3.

Carry out the monitoring by using the hard surface swab sampler, covering the 10 cm x 10 cm surface sampling with a crossed zigzag. Complete the first zigzag with firm pressure; the second zigzag draw

will be smoother and gentler to recover all the potential contaminants. See Figure 4.

It is recommended to delimit the area to be sampled using a template to ensure that the mentioned surface is taken in a more consistent sampling. If it is possible, mark it on the table surface so you are able to see through it and follow it as a reference. Take the swab sampler and introduce it into the AccuPoint Advanced reader, close it, and wait to see the results on the screen.

A preliminary proof of concept sampling using at least three different brands of food wrap-up plastic was sampled 10 times per brand to demonstrate that a signal of zero "0" RLU's was achieved by sampling the sticky inner surface of the plastic.

A broiler breeder company will have two different intervention protocols based on two groups of collected fertile eggs; one that is coming from the nest and the other experiencing floor contact or with any visual organic matter present at the egg collection time. The nest group was under a gas-based control enclosure formaldehyde exposure, and the floor (dirty) egg group was treated with a coarse-spray peracetic solution at 37 Celsius. This company allowed the sampling collection for six consecutive weeks. Each day, five random egg trays were sampled from each group, for a total of 25 samples obtained per group per week. Seven hundred fifty eggs per week per group were under the screening hygiene monitoring process. After more than six weeks of continuous monitoring, more than 4,500 eggs per group were screened.

RESULTS

The sampling protocol was able to demonstrate a clear separation of the two intervention protocols, revealing consistent lower RLU's for the group of eggs that were treated directly over the eggshell surfaces with a sanitizer. See Graph 1.

DISCUSSION

It is considered that this sampling method could show a trend in the results. It is assumed that there is not a total transfer of the contamination present in the shell and the result indicates the relative light units (RLU) associated with the presence of potential contaminants that may include bacteria and/or organic matter.

This study proposes an innovative way of assessing the potential relative amount of present contaminants on eggshells without inducing potential cross-contaminations that could be caused by the protocol itself.

REFERENCES

1. Tetro, Jason A and Syed A. Sattar, The Application of ATP Bioluminescence for Rapid Monitoring of Microbiological Contamination on Environmental Surfaces: A Critical Review, Infection Control Tips (2021)

2. Whiteley, G.S et al. A suggested sampling algorithm for use with ATP testing in cleanliness measurement. Infection Disease & Health (2016) 21: 169-175

3. National Hog Farmer. ATP bioluminescence effective cleanliness assessment tool (2016)

4. Munoz, R et al. Monitoring and auditing a biosecurity program with AccuPoint Advance. 65th Western Poultry Disease Conference p 187-188 (2016)

5. Thiemann, Rachel et al. Comparison of ATP Bioluminescence to traditional colony counting for bacterial enumeration of treated and non-treated hatching eggs. Proceedings of Southern Conference on Avian Diseases 63rd annual meeting (2022).

Figure 1.

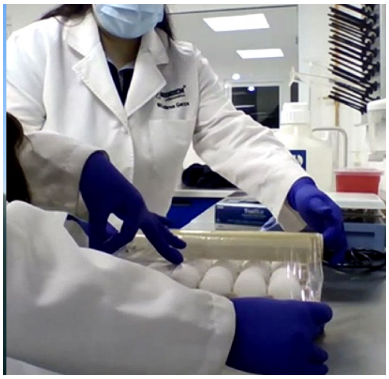
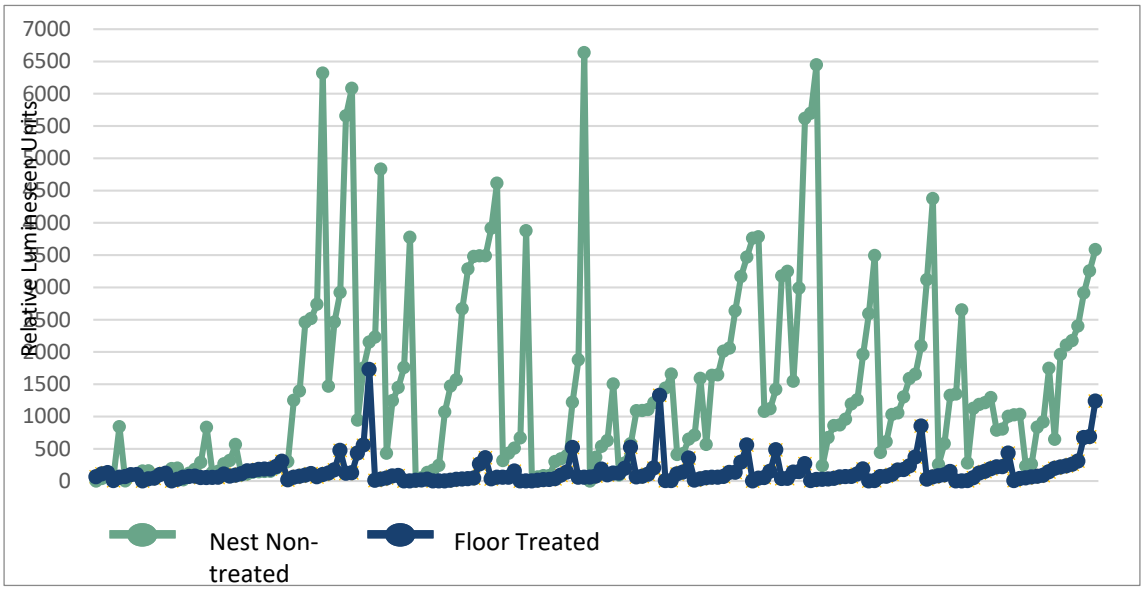


Figure 2.

Figure 3.

Figure 4.

Graph 1. Results.



BAD BLOOD: USING A MODERN TOOL TO AID IN POULTRY DISEASE DIAGNOSTICS

J. Nicholds[A], E. Shepherd[A], K. Grogan[A], D. French[A], and T. Gamble[B]

[A]Department of Population Health, Poultry Diagnostic and Research Center, College of Veterinary Medicine, University of Georgia, 953 College Station Road, Athens, GA 30602
[B]Pilgrim's, 1770 Promontory Circle, Greeley, CO 80634

SUMMARY

Blood biochemistry analysis is an excellent tool to help characterize the homeostatic state, or lack thereof, that a bird is experiencing. In recent years, avian researchers and clinicians have made strides to improve the diagnostic capabilities for understanding metabolic status in poultry species. Our team has been using the i-STAT® Alinity V (Zoetis, Parsipanny, NJ) portable clinical analyzer in the field and in the diagnostic lab as an aid in the diagnostic process. The details of two clinical presentations in broiler chicks and the context of the i-STAT findings will be reviewed. The first clinical presentation is of hypocalcemia in broilers and the second presentation is of sodium toxicity, both during the first week of age.

CASE 1

Introduction. In January of 2021, 12 live and five dead seven-day-old broilers were submitted to the Poultry Diagnostic and Research Centers (PDRC) Diagnostic lab for necropsy and a request to evaluate for hypoglycemia. Additional submissions from the same integrator were also subsequently received, all with similar history and findings. This company was observing increased first week mortality and identifying birds between two to seven days of age found in sternal recumbency with extended legs and neck. Some birds appeared blind, had muscle fasciculations and/or head tremors. Birds with this presentation typically died in under 30 minutes. All houses on an affected farm were usually affected and the prevalence varied from 0.5-10%. Symptoms and mortality with this presentation typically ceased by 10-12 days of age. Seven complexes reported seeing affected birds and while cases dwindled during the summer months, they increased again with winter weather late in 2021 and into 2022.

Results and discussion. Blood was collected from live birds for biochemical evaluation using the i-STAT Alinity V portable clinical analyzer and CG8+ cartridges (Zoetis, Parsipanny, NJ). The CG8+ cartridge provides values for sodium (Na mmol/L), potassium (K mmol/L), ionized calcium (iCa mmol/L), glucose (Glu mmol/dL), hematocrit (Hct, packed cell volume [PCV]), pH, partial pressure carbon dioxide (PCO2 mm Hg), partial pressure oxygen (PO2 mm Hg), and bicarbonate (HCO_3^- mmol/L). A consistent result from clinically affected birds was low iCa, ranging from 0.49-1.56 mmol/L with an average of 0.77 mmol/L (n=23). Clinically normal appearing birds in the same submissions ranged from 0.82-1.4 mmol/L with an average of 1.22 mmol/L (n=12). Published normal ranges for ionized calcium in poultry species vary from narrow, 1.35-1.55 mmol/L (1), to broad 1.2-1.73mmol/L (2). The iCa values from birds with symptoms in the affected flocks were well below these ranges and birds without symptoms from within the same flock were low to marginal.

Necropsy findings from birds with this presentation included mild to moderate gizzard erosions and proventriculitis as well as subjectively soft bones with widened growth plates. Extensive histologic evaluation was conducted. There were no consistent lesions to suggest infectious etiology and no lesions to suggest liver or kidney damage, which might indirectly impact calcium homeostasis. Extensive additional diagnostic work up was completed at PDRC and other diagnostic labs, still yielding no definitive diagnosis and ruling out many other differential diagnoses.

We are confident that the clinical signs in these cases are the result of acute hypocalcemia, manifesting as tetany. The cause of hypocalcemia has yet to be determined.

CASE 2

Introduction. In October of 2021, a submission from the same integrator as Case 1, of 11 live six-day old broilers and a feed sample were received by PDRC's diagnostic lab. The presenting complaint was high mortality and birds found in sternal recumbency with drooped heads. Additional submissions from the same integrator and complex location were also subsequently received.

Results and discussion. Given the age and presenting complaint, blood samples were collected

from live birds for biochemical evaluation using the i-STAT Alinity V portable clinical analyzer to rule out potential hypocalcemia. Upon examination and manipulation for blood sampling, mild to severe head tremors were noted. Reviewing the i-STAT CG8+ cartridge results during the course of necropsy it was noted that 5/5 birds sampled had Na values above the limit of detection (180mmol/L) for the i-STAT. A range for blood sodium in poultry species is 146-169 mmol/L (1). This immediate diagnostic result moved sodium toxicity to the top of the differential list. Subsequent histologic findings in brain tissue included encephalopathy with intracellular and perivascular edema. Brain sodium from a pooled sample tested at 1968 ppm sodium. The toxic threshold for brain sodium in poultry has been reported at 1900 ppm (3), and >2000 ppm is considered diagnostic for sodium toxicity in cattle and swine (4, personal communication). A case report of sodium toxicity in commercial turkeys (5) documented brain sodium levels at 1870-2680 ppm and noted an average brain sodium of 1233 ppm in turkeys <7 days of age submitted for other reasons. The feed sample in this case was within expected range for sodium at 1694 ppm (as sampled). Together, the iStat results, histopathology findings, and brain sodium levels led to a conclusion of sodium toxicity. Subsequent submissions of similar age birds and feed from this same complex with similar presenting complaint were also evaluated. In two of these cases, feed sodium tested at 3009 ppm and 7792 ppm, and in the latter case, brain sodium levels were 2451 ppm. These results further supported the initial conclusion of sodium toxicity.

CONCLUSIONS

These case presentations illustrate that handheld clinical analyzers can play a critical role in the pursuit of a diagnosis in certain clinical poultry case presentations. A clinician must use their knowledge and clinical judgement to determine if use of the handheld device is warranted and/or prudent. The data in the cases presented confirmed a suspicion of calcium tetany and rapidly diagnosed a sodium toxicity. In the case of sodium toxicity, the diagnostic information was delivered the same day as the necropsy submission was made which allowed for swift and definitive follow up and investigation into contributing factors. The ancillary diagnostics, histopathology, brain sodium levels and feed testing which supported the initial finding took 4, 11 and 21 days respectively. Handheld analyzers have the potential to assist with rapid diagnosis and potential treatment in select poultry case presentations. A growing need is the establishment of appropriate reference ranges against which to interpret findings.

REFERENCES

1. Chorfi, Y. and D. Venne. Blood Biochemistry in Birds. In: Manual of Poultry Diseases. J. Burgere-Picoux, J-P. Vaillancourt, H. L. Shivaprasad, D. Venne, and M. Bouzouaia ed. Association francaise pour l'avancement des sciences, Paris, Franc. pp. 80-86. 2015.

2. Martin, M. P., M. Wineland, and H. J. Barnes. Selected Blood Chemistry and Gas Reference Ranges for Broiler Breeders Using the i-STAT® Handheld Clinical Analyzer. Avian Dis. 54:1016-1020. 2010

3. Himsworth, C. Salt toxicity in broiler breeder chicks. Oral presentation at the Western Conference of Veterinary Diagnostic Pathology. 2015.

4. Buchweitz, J. P. Clinical Toxicologist. Michigan State University, Veterinary Diagnostic Laboratory. 2021.

5. Crespo et al. Bilateral Malacia Associated with Sodium Poisoning in Turkey Poults. Avian Dis 2008; 52:179-192.

RE-EMERGENCE OF INCLUSION BODY HEPATITIS IN CANADA

D. Ojkic and E. Martin

Animal Health Laboratory, University of Guelph, Guelph, Ontario Canada

SUMMARY

Inclusion body hepatitis (IBH) caused by fowl adenovirus (FADV) infection can be a devastating disease of broilers resulting in significant economic damage to poultry producers. From 2000-2006 approximately 40% of IBH cases in Ontario and Canada were caused by FADV11, 40% by FADV8a and remaining 20% involved FADV8b and FADV2 (1). In 2010 a wide-spread vaccination program of broiler-breeders was introduced in Ontario with autogenous FAdV8a/FAdV11 vaccines and in years following the introduction of vaccination the incidence of IBH was decreasing. However, since 2014 the identity of FAdVs involved in IBH has been shifting and the frequency of IBH submissions has been increasing. Results of hexon-based genotyping of 592 diagnostic samples from 2014 -2021 show that the increase in IBH incidence has been primarily associated with FAdV8b infection. By 2021 FAdV8b has been detected in around 90% of all IBH-related diagnostic submissions almost completely displacing FAdV8a and FAdV11.

(The full-length article will be published in *Avian Pathology*.)

REFERENCES

1. Ojkic D, Martin E, Swinton J, Vaillancourt JP, Boulianne M, Gomis S. Genotyping of Canadian isolates of fowl adenoviruses. Avian Pathol. 2008 Feb;37(1):95-100.

SALMONELLA INFECTION IN CHICKENS AND PERSISTENCE IN POULTRY ENVIRONMENTS

M. Pulido Landinez

Associate Clinical Professor, Poultry Research and Diagnostic Laboratory, College of Veterinary Medicine, Mississippi State University

INTRODUCTION

Among the current challenges for poultry production, *Salmonella* (S) presence in chickens implies a high risk in terms of public health. Occasionally, some *Salmonella* serotypes can cause severe disease and mortality in chickens one to two weeks old, among them *S*. Enteritidis, and recently observed *S*. Infantis, both acting mainly as secondary bacteria. Furthermore, antimicrobial resistance exhibited by *Salmonella* raises another concern.

Salmonella is a problem of the whole chicken meat supply chain. Its control in primary production is crucial! Each sector on the vertical integration must develop its *Salmonella* interventions considering all subsequent sectors, to respond to the consumer`s demands.

Understanding how *Salmonella* adapts to chickens and poultry environments, and what mechanisms these bacteria use to persist can help make decisions about control measures to implement. Additionally, this knowledge will allow establishing a routine evaluation of the interventions used to control this bacterium.

Control of specific *Salmonella* serotypes: resident vs. transitory serotypes. The specific diagnosis of the predominant *Salmonella* serotype is essential in terms of control. Many decisions are made by knowing which are the predominant serotypes. The best example is the Salmonella vaccination plan. However, knowing what serotypes are predominant, allows us to understand why *Salmonella* can become persistent in a chicken farm since some serotypes can adapt better to the chickens and their environment than others.

Recently, we introduced the concept "*Salmonella* resident serotypes" and the importance of their early identification to be successful in *Salmonella* control. While some Salmonella serotypes can be considered as transient organisms, being unable to persist in the chickens and their environment, other serotypes can get completely established in poultry facilities. Resident *Salmonella* serotypes can grow and create bacterial communities in the chickens and their environment. These "resident serotypes" persist a long time, even after strict sanitation processes (Table 1).

The worst scenario occurs when *Salmonella* serotypes with high importance in terms of public health (Enteritidis, Heidelberg, Typhimurium, and Infantis, among others); can persist in broiler vertical integrations. Currently, antimicrobial resistance exhibited by some serotypes with minimum effect in terms of public health as Kentucky constitutes another important concern.

In recent years, *Salmonella* Infantis (SI) has become one of the most frequently *Salmonella* serotypes isolated from poultry and their products. Outbreaks of foodborne illness involving *S*. Infantis are being common in the United States, Canada, and Europe. Furthermore, this serotype has shown a marked multi-resistance to antibiotics.

Analysis of *S*. Infantis isolation data in two regions of South America showed that in addition to the findings in the processing plants, the presence of this serotype in samples collected from grandparent and breeder farms, hatcheries (PIPS), and day-old chicks is frequent. These findings suggest this serotype may be transmitted vertically or by the contamination of the eggshell. It is also possible that this serotype is adapted to the egg (Pulido-Landinez *et al*., 2021). Analyzing special plasmids present in these *S*. Infantis isolates, Sanchez-Ingunza *et al*. (2020) identified special traits conferring resistance to specific disinfectants as quaternary ammonia. Studies of *S*. Infantis survival in litter and hatchery residues from Mississippi broiler integrations showed that under special conditions this *Salmonella* serotype can survive up to 100 days (Table 2).

Are the resident serotypes the reason why is so difficult to keep *Salmonella* numbers low? This is a current powerful USDA recommendation in terms of *Salmonella* control (https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/defend-the-flock-program). Knowing how hard is to get *Salmonella* zero in the poultry industry, the current goal is to decrease *Salmonella* loads in each sector of the vertical integration. Therefore, primary production will contribute to having very low *Salmonella* loads in the chicken carcass and parts. Each one must do its part! Examples of salmonella's ability to survive and persist in poultry materials are presented in Table 2.

How does *Salmonella* gets adapted to the poultry environments? Numerous studies have been performed in the attempt to understand how *Salmonella* gets adapted to the chickens and poultry environments. This bacterium has multiple mechanisms allowing it to survive and multiply in a very efficient way. Based on the results of different studies, it is possible to say that *Salmonella* is exceptionally resourceful in getting adapted to the poultry environment. Ten of these mechanisms are presented below:

1. *Salmonella* bacteria have effective systems to adhere to gut cells. *Salmonella* multiplication begins after the invasion of the intestinal epithelial cell.
2. *Salmonella* bacteria interact with resident microbiota. Controlling both, the microbiota in the gut and the one in the environment is important.
3. High-temperature tolerance. Some *Salmonella* serotypes can survive after pelleting and litter treatment.
4. Adaptation to acid conditions. If *Salmonella* bacteria exhibit this characteristic, control using acids will not work well.
5. Biofilm production. This is the most common tool used by *Salmonella* to avoid disinfectants action. A good cleaning is imperative before disinfection.
6. Iron acquisition: High or fluctuant levels of Iron in the drinking water will promote *Salmonella* persistence in the chickens, plagues, and environment.
7. Continuous passage through chickens. Chickens can constantly acquire *Salmonella* through the feces.
8. Carriers. The most important *Salmonella* carrier is the chicken itself. However, plagues as mice, flies, and dark beetles can act as carriers, too.
9. Constant multiplication and persistence in plagues. *Salmonella* presence in plagues has been identified as one of the most important sources of *Salmonella* in consecutive flocks.
10. Skin binding tools. *Salmonella* bacteria can attach to the chicken's skin and feather follicles. *Salmonella* in the litter increases the chances of finding *Salmonella* in the chicken carcass and parts at processing.

Recent studies performed by Matera *et al*. (2022) are highlighting an interesting *Salmonella* adaptation method. Based on the concept that the envelope of Gram-negative bacteria is a vital barrier that must balance protection and nutrient uptake, this study talks about the presence of "sponges" in the outer membrane. Such sponges belong to the class of "small RNAs." Small RNAs are crucial regulators of bacterial envelope composition and function. The RNAsponge OppX mimics the actual binding target of a special sRNA, the so-called MicF sRNA, in the bacterial outer membrane, intercepting it before it reaches its destination. Or in other words, it absorbs it like a sponge. If the bacterium lacks the OppX sponge, its growth will be restricted, especially in a nutrient-poor environment. If, however, enough OppX is available, the OmpF pores in the membrane also become more active, increasing the uptake of nutrients if they are scarce. This mechanism may be allowing *Salmonella* to survive under challenging conditions.

REFERENCES

1. Álvarez-Ordóñez, A., Fernández, A., López, M. 2009. Comparison of acids on the induction of an Acid Tolerance Response in Salmonella typhimurium, consequences for food safety. Meat Science 81:65–70

2. Álvarez-Ordóñez, A., Prieto, M., Bernardo, A., Hill, C., López, M. 2012. The Acid Tolerance Response of Salmonella spp.: An adaptive strategy to survive in stressful environments prevailing in foods and the host. Food Research International 45: 482–492.

3. Aviv G, Rahav G, Gal-Mor O. 2016. Horizontal transfer of the Salmonella enterica serovar Infantis resistance and virulence plasmid pESI to the gut microbiota of warm-blooded hosts. mBio 7(5):e01395-16. doi:10.1128/mBio.01395-16.

4. Burgess, C., Gianotti, A. Gruzdev, N., Holah, J., Knøchel, S. Lehner A., Margas, A., Schmitz, S., Shlomo . E., Saldinger., E., Tresse. O. 2016. The response of foodborne pathogens to osmotic and desiccation stresses in the food chain. International Journal of Food Microbiology 221: 37–53. ISSN 0168-1605.
https://doi.org/10.1016/j.ijfoodmicro.2015.12.014.

5. CDC. 2018. Outbreak of Multidrug-Resistant Salmonella Infections Linked to Raw Chicken Products Available at https://www.cdc.gov/salmonella/infantis-10-18/index.html

6. Franco A, Leekitcharoenphon P, Feltrin F, Alba P, Cordaro G, Iurescia M, et al. 2015. Emergence of a Clonal Lineage of Multidrug- Resistant ESBL-Producing Salmonella Infantis Transmitted from Broilers and Broiler Meat to Humans in Italy between 2011 and 2014. PLoS ONE 10(12): e0144802. doi:10.1371/journal.pone.0144802

7. Finn, S., Condell, o., McClure, P., Amézquita, A., Fanning, S. 2013. Mechanisms of survival, responses, and sources of Salmonella in low-moisture environments. Front. Microbiol., 4(331): 1-15. https://doi.org/10.3389/fmicb.2013.00331.

8. Gruzdev, N., Pinto, R., Sela, S. 2011. Effect of Desiccation on Tolerance of Salmonella enterica to Multiple Stresses. Applied and Environmental Microbiology. 77(5): p. 1667–1673. doi:10.1128/AEM.02156-10.

9. Matera et al., 2022. Global RNA interactome of Salmonella discovers a 50 UTR sponge for the MicF small RNA that connects membrane permeability to transport capacity Molecular Cell 82, 629–644. https://doi.org/10.1016/j.molcel.2021.12.030

10. Medina-Santana, J; Ortega-Paredes, D; De Janon, S; Burnett, E; Ishida, M; Sauders, B; Stevens, M; Vinueza-Burgos C. Investigating the dynamics of Salmonella contamination in integrated poultry companies using a whole-genome sequencing approach. 2022 Poultry Science 101:101611. https://doi.org/10.1016/j.psj.2021.101611

11. Pulido-Landínez, M., R. Sanchez-Ingunza, J. Guard, and V. P. do Nascimento Assignment of serotype to Salmonella enterica isolates obtained from poultry and their environment in southern Brazil. Lett. Appl. Microbiol. 57:288-294. 2013.

12. Pulido-Landínez, M., P. Washington, J.K. Thornton, Y. Zhang, R. Sanchez-Ingunza, A. Banda, J. Guard, and V. P. do Nascimento, D. L. Magee, and M.J. Mauel Serotype and Antimicrobial Resistance Patterns of Salmonella Isolates from Commercial Birds and Poultry Environment in Mississippi. Avian Diseases 58:64-70. 2014.

13. Pulido Landinez, M. Basic concepts for the control of Salmonella in Colombian poultry. (Book written in Spanish: Conceptos básicos para el control de Salmonella en la Avicultura Colombiana. Colombian Poultry Producers Association. 104 pp. ISBN 978-958-59990-8-4. 2018.

14. Pulido Landinez, M. Food safety - Salmonella update in broilers. Animal Feed Science and Technology 250 (2019) 53–58. https://doi.org/10.1016/j.anifeedsci.2019.01.008.

15. Sánchez-Ingunza, R. and Pulido-Landinez, M. Comparative Analysis of the Salmonella enterica subsp. enterica serovar Infantis IncFIB Plasmid. Oral presentation at AAAP/AVMA Annual Meeting. August 2020. Virtual meeting.

15. Mauri-Villarreal, Y; Paredes-Barbosa, R; and Pulido-Landinez, M. Salmonella diversity and antimicrobial resistance behavior: the feed mill a source? T41. Metabolism and Nutrition: Enzymes, Feed Additives. International Poultry Scientific Forum. US Poultry and Egg Association. January 26-27. 2021. Atlanta, GA. Proceedings at: https://www.ippexpo.org/ipsf/docs/IPSF-SPSS-Abstracts-2021.pdf

16. Pulido-Landinez, M., Rocio Paredes-Barbosa, Yuli Melisa Sierra-Arguello. The Role of Vertical Transmission and Egg Adaptation of Salmonella Infantis in Broiler Vertical Integrations. Oral presentation at AAAP/AVMA Annual Meeting. July 30-August 2, 2021. Virtual meeting. Proceedings at: https://aaap.memberclicks.net/assets/2021_Annual_Meeting/AAAP_2021_Proceedings.pdf

Table 1. *Salmonella* transitory vs. resident serotypes characteristics.

Transitory	Residents
• Occasionally isolated and identified. • Many are not pathogenic. • Can be removed with routine cleaning and disinfection procedures. • Must be identified and prevented from adapting to the chickens and poultry environments.	• Could be fully established (adapted) in chickens and poultry environment. • Multiplication is mostly exponential (one *Salmonella* cell can produce many *Salmonella* bacteria colonies). • Could be fully adapted to the plagues present in the chicken house. • Persist for a long time. • Routine cleaning and disinfection procedures do not remove them. • Present in consecutive flocks

Table 2. *Salmonella* survival in material and sources related to poultry farms.

***Salmonella* serotype-source**	**Survival time**	**Reference**

S. Enteritidis and insects:
- Cockroaches
- Flies
  - External Surface
  - Intestines
- Black beetles (Larvae)
- Fleas parasitizing mice
	
	17 d
	
	24h
	7d -28d
	240 d
	All flea's life
		Krieg et al 1959, Eskey et al 1949, Ostrolenk and Welch, 1942, cited by Mitscherlich y Marth, 2012. Davies, 2003. Pulido-Landinez, 2018.

S. Enteritidis in feces of rats, foxes, and cats	240 d	Davies, 2003. Pulido-Landinez, 2018.
S. Enteritidis in litter	240 d	Davies 2003. Pulido-Landinez, 2018.
S. Enteritidis in commercial layer houses. - Litter (depending on water activity and pH) consecutive flocks	780 d	DAERA, 2012, 1995, Pulido, 2015. SAG Chile, 2016. Dunlop et al 2016. Pulido-Landinez, 2018.
S. Typhimurium: - Feed - Litter - 77°F.	 480 d 540 d	SAG Chile, 2016. Pulido-Landinez, 2018.

S. Infantis
- Litter - 70°F
- Litter - 39°F
- Hatchery residues: under different light and temperature conditions
  - Dark - 68°F
  - Warm - 79°F
  - Cold - 39°F
	
	Up to 30 d
	Up to 35 d
	
	Up to 32 d
	Up to 32 d
	Up to 100 d
		Pulido-Landinez and Miller 2020 (unpublish data).
		
		Pulido-Landinez et al 2020

RETROSPECTIVE REVIEW OF PIGEON PARAMYXOVIRUS-1 LAB SUBMISSIONS FROM PEN-REARED PIGEONS IN CALIFORNIA: 2010 - 2020

S. Ramsubeik[A], C. Jerry[A], H. L. Shivaprasad[B] , B. Crossley[C] , and S. Stoute[AD]

[A]Turlock Branch, University of California, Davis, California Animal Health and Food Safety Laboratory System, 1550 N. Soderquist Road, P.O Box 1522, Turlock, CA 95380
[B]Tulare Branch, University of California, Davis, California Animal Health and Food Safety Laboratory System, 18760 Road 112, Tulare, CA 93274
[C]University of California Davis, School of Veterinary Medicine, 1089 Veterinary Medicine Drive, Vet Med 3B Room 4007, Davis, CA, 95616

SUMMARY

Pigeon paramyxovirus-1 (PPMV-1) is a virulent member of the Avulavirus family, which can result in elevated mortality and economic losses to pigeon lofts. California is a large squab-producing region in the United States, and has well-established racing and show pigeon clubs and events throughout the State. A retrospective analysis of pigeon necropsy submissions to the California Animal Health and Food Safety Laboratory System from 2010 to 2020 revealed 23% (53/230) of submissions diagnosed with PPMV-1. In addition, the highest frequency of PPMV-1 for the period occurred during the spring season in 36.1% (22/61) and 2014 had the highest percentages of cases detected (41.4%, 5/12). The positive cases comprised squabs (25.9%, 28/108), and racing and show pigeons (20.5%, 25/122). Juvenile and adult pigeons were submitted with a history of increased mortality, neurologic signs, diarrhea and weight loss. The most consistent macroscopic findings were enlarged, pale kidneys, pale or mottled pancreas and enlarged spleens. The most common microscopic findings were observed in the kidney, pancreas, brain and spleen.

INTRODUCTION

Pigeon paramyxovirus-1 (PPMV-1) is a virulent member of the Avulavirus family, which is host adapted to Columbiformes birds (1, 2). The virus causes mortality and economic losses to commercial pigeon operations globally. PPMV-1 is believed to be endemic in Columbiformes in the US and widespread in racing pigeon lofts (1, 3, 4).

California is a large squab-producing region in the United States, and has well-established racing and show pigeon clubs and events throughout the State (3, 5, 6). The objectives of this study were to determine the current prevalence of PPMV-1 in squabs and other commercial pigeons in California, identify epidemiological trends and determine the current presentation of the disease based on evaluation of pigeons cases submitted to the CAHFS diagnostic lab. These findings would further assist in understanding, diagnosing and preventing PPMV-1 infections in regions with intensive commercial pigeon populations.

MATERIALS AND METHODS

The electronic database at the California Animal Health and Food Safety (CAHFS) laboratory system was searched for commercial pigeon necropsy cases in which PPMV-1 was diagnosed for the period January 1st 2010 to December 31st 2020. Data analyzed for each case included date of submissions, clinical signs (clinical presentation when birds were submitted live or as specified in the history), macroscopic lesions, microscopic lesions, flock location, vaccination history, laboratory tests and concurrent disease conditions. Repeated flock submissions were excluded from the dataset. Commercial pigeons were defined as squab or other (racing, fancy or show breeders).

PPMV-1 was assigned as the definitive case diagnosis based on the clinical findings, gross lesions, microscopic lesions and vaccination history (if applicable) coupled with positive semi quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay, virus isolation and/or genetic sequencing of the virus and/or positive paramyxovirus 1 immunohistochemistry as previously described (6).

The study analyzed commercial pigeon necropsy submissions in which a routine postmortem examination was conducted and gross anatomic findings were recorded. Gross and histologic lesions associated with the brain, kidney, spleen, pancreas and intestines were analyzed. For histopathology, sections were collected and fixed in 10% neutral-buffered formalin, processed routinely, embedded in paraffin,

sectioned at 4 µm, stained with hematoxylin and eosin and examined by bright field microscopy.

The difference in prevalence of PPMV-1 with factors such as age, seasonality and pigeon type were assessed using the Chi-Square test of independence or Fisher's exact test. *P* values ≤ 0.05 were considered statistically significant. Analysis was performed using the statistical software package STATA (Stata Corp, College Station, TX).

RESULTS

Commercial pigeon submissions for which PPMV-1 diagnostics were performed from January 1st 2010 to December 31st 2020 totaled 230; with PPMV-1 diagnosed in 23% (53/230)) cases involving 206 pigeons. PPMV-1 was diagnosed in 25.9% (28/108) of squab submissions and 20.5% (25/122) of cases classified as others.

For the study period analyzed, PPMV-1 was diagnosed throughout the years with the highest percentage occurrence in 2014 (41.7%). During the period, PPMV-1 disease occurrence was detected in all seasons of the year with the highest prevalence in the spring months (36.1%, p =0.006).

Client complaint on submissions included increase mortality, birds exhibiting neurologic signs (torticollis, head tremors, wing droop and leg paralysis), diarrhea and weight loss. Of the 53 cases diagnosed with PPMV-1 infection neurological signs, emaciation, lethargy and diarrhea were the predominant clinical signs noted at necropsy. The most common macroscopic and microscopic findings in PPMV-1 diagnosed cases are listed in Table 1 and Table 2 respectively.

DISCUSSION AND CONCLUSION

This study was based on data obtained for the past 11 years (2010 to 2020). During the period, 23% of commercial pigeon flocks were found to be affected by PPMV-1. The percentage of PPMV-1cases was greater in meat squab submissions compared to racing or show pigeons, however there was no statistically significant difference in prevalence based on pigeon type (p> 0.05). The disease is host adapted to pigeons and other Columbiforms, and is believed to be maintained in pigeons raised in squab facilities and in private lofts such as racing and show pigeons (Brown 2017). Transmission of PPMV-1 may occurs due to a breakdown in biosecurity practice, movement of squab during processing and shows or racing events and practices. (Brown, Barton, Alexander).

During the review period, PPMV-1 disease occurrence was detected in all seasons of the year, with the highest prevalence in the spring months. Possible reasons for increased disease outbreaks during the spring season can be linked to breaches in biosecurity, with increased movement and activity. Clinical, gross and microscopic findings were consistent with those reported in previous studies (3, 6, 7). This study reports on the current prevalence of PPMV-1 in commercial pigeons in California and it provides an update on PPMV-1 disease, as there have been changes in the production landscape, vaccination and prevention strategies and improvements in PPMV-1 disease diagnostics.

(The full-length article will be published in *The Journal of Applied Poultry Research*.)

REFERENCES

1. Alexander DJ, Wilson GW, Thain JA, Lister SA. Avian paramyxovirus type 1 infection of racing pigeons: 3 epizootiological considerations. *Vet Rec.* 115:213-6; eng. 1984 Sep 1.

2. Brown VR, Bevins SN. A review of virulent Newcastle disease viruses in the United States and the role of wild birds in viral persistence and spread. *Vet Res.* 48:68; 2017 Oct 26.

3. Barton JT BA, Cooper GL, Charlton BR, Cardona CJ. Avian Paramyxovirus Type 1 Infections in Racing Pigeons in California. I. Clinical Signs, Pathology, and Serology. *Avian Dis.* 36:463-468; 1992.

4. Kim LM, King DJ, Guzman H, Tesh RB, Travassos da Rosa AP, Bueno R, Jr., Dennett JA, Afonso CL. Biological and phylogenetic characterization of pigeon paramyxovirus serotype 1 circulating in wild North American pigeons and doves. *J Clin Microbiol.* 46:3303-10; 2008 Oct.

5. Isidoro-Ayza M, Afonso CL, Stanton JB, Knowles S, Ip HS, White CL, Fenton H, Ruder MG, Dolinski AC, Lankton J. Natural Infections With Pigeon Paramyxovirus Serotype 1: Pathologic Changes in Eurasian Collared-Doves (Streptopelia decaocto) and Rock Pigeons (Columba livia) in the United States. *Vet Pathol.* 54:695-703; 2017 Jul.

6. Rogers KH, Mete A, Ip HS, Torchetti MK, Killian ML, Crossley B. Emergence and molecular characterization of pigeon Paramyxovirus-1 in non-native Eurasian collared doves (Streptopelia decaocto) in California, USA. *Infect Genet Evol.* 91:104809; 2021 Jul.

7. Tangredi BP. Avian paramyxovirus type 1 infection in pigeons: recent changes in clinical observations. *Avian Dis.* 32:839-41; eng. 1988 Oct-Dec.

Table 1. Gross lesion observed in PPMV-1 diagnosed cases.

Gross lesions	Percent (No. of cases/ total cases)
Enlarged/pale/mottled kidneys, urates	50.9 (27/53)
Enlarged/pale/mottled pancreas	37.7 (20/53)
Enlarged/ mottled spleens	34.0 (18/53)
Greenish watery intestinal content	15.1 (8/53)

Table 2. Microscopic lesion observed in PPMV-1 diagnosed cases.

Histologic lesions	%age (# of cases/total cases)
Lymphoplasmacytic interstitial nephritis	79.2 (42/53)
Lymphoplasmacytic pancreatitis	64.1 (34/53)
Gliosis/lymphocytic perivascular cuffing, encephalomyelitis	47.2 (25/53)
Lymphofollicular splenitis	18.9 (10/53)

PREVALENCE AND VIRAL ECOLOGY OF AVIAN INFLUENZA IN WETLANDS OF HIGH VERSUS LOW WATERFOWL USE IN THE CENTRAL VALLEY OF CALIFORNIA

B. Riggs[A], S. Díaz-Muñoz[B], E. Matchett[C], and M. Pitesky[A]

[A] Department of Population Health and Reproduction, School of Veterinary Medicine – Cooperative Extension, University of California Davis, Davis, California 95616.
[B]Department of Microbiology and Molecular Genetics, University of California, Davis, Davis, California 95616
[C]U.S. Geological Survey, Western Ecological Research Center, Dixon Field Station, Suite D Dixon, California, USA.

ABSTRACT

Exposure to avian influenza (AI) is a significant concern to the commercial poultry industry. Waterfowl are the primary reservoir of the virus, therefore understanding the presence/absence and type of AI in waterfowl and their habitat (e.g. soil and water associated with wetlands) is essential toward mitigating risk. Here we compare the presence/absence and viral ecology of AI in wetlands with both high and low waterfowl density as determined via a combination of the California Waterfowl Tracker (CWT) and knowledge/observation. Specifically, a total of 182 oropharyngeal/cloacal samples, as well as 100 sediment samples, and 120 water samples were collected, amplified, and sequenced during the summer of 2021. Results will be used to provide a new layer of data for the CWT which allows stakeholders to identify the presence/absence of waterfowl near their farms. With this new layer of data, the CWT will be more applicable and accessible to poultry producers throughout the central valley of California.

INTRODUCTION

Migratory waterfowl, particularly those of the family *Anatidae*, have been found to represent an asymptomatic, naturally occurring reservoir for avian influenza viruses (AIV), which can be spread to commercial poultry (1). Although the National Poultry Improvement Plan (NPIP) has an AIV surveillance system in place for domestic poultry, there is currently no analogous system in migratory waterfowl, which is the primary reservoir of the virus. In addition, as the primary reservoir, waterfowl are considered the primary source of novel strains of AIV due to the crossover of migratory routes and the common exchange of the virus from bird to bird (2).

Commercial poultry are highly susceptible to both high-pathogenic and low-pathogenic strains of AIV (3). In 2020-2021 alone, approximately 22,900,000 affected poultry were reported in 31 European countries, as well as 3,777 reported highly-pathogenic infected premises (4). Even with heightened biosecurity, AIV can still spread into a commercial poultry facility. Both local and migratory waterfowl can congregate near or on commercial poultry facilities; current surveillance is limited and poorly integrated regionally and nationally (5).

Remote surveillance of waterfowl offers a novel method of understanding potential risk of disease transmission. Applications such as the CWT, which utilize remote technologies including radar, satellite imagery, and weather station data to model waterfowl density could be useful as a real-time tool for commercial producers (6). As an example, 11 GPS-tagged ducks between 2015-2020, were observed to have spent substantial amounts of time near (i.e. within 10 km) or on a commercial livestock or poultry facility (7).

In the central valley of California, peak migrations of waterfowl occur between late fall and early spring as part of the normal migratory path of the Pacific Flyway (8). Next generation surveillance tools such as the CWT could be used to target testing of the environment and waterfowl for AIV. Here we describe the sampling of high and low waterfowl density wetlands, in order to better understand the relationship between the presence/absence of waterfowl, and AIV in waterfowl, compared to the viral ecology of AIV in wetland sediment and water. Sediment and wetland water filtration methods developed by McCuen *et al*. (9), were used to compare viral prevalence and ecology between high and low use wetlands and their associated waterfowl, in order to test the hypothesis that the AIV load and diversity is significantly higher in ponds of high use versus low use.

MATERIALS AND METHODS

Wetland selection criteria. Water samples for the pre-migration time frame were collected in August and September 2021, from 10 individual wetland ponds from each of two watershed basins (five high-use and five low-use), totaling 20 wetland ponds identified for sampling (Figure 1). Within each of the two basins (specifically Sacramento National Wildlife Refuge Complex and the California Butte Sink State Wildlife Areas), locations were chosen due to waterfowl use within each individual pond. In 2021, ponds were identified for sampling using telemetry data (home ranges of ducks in summer and winter) and satellite imagery that indicated habitats that were flooded from late July to early September 2021. Additional insights were gained from the U.S. wildlife refuge and state wildlife area staff.

The selected wetlands were free of chemical treatments of any kind, in order to represent a natural habitat for waterfowl. Potential salinity was also a factor in determining sampling location, as AIV prevalence is typically lower in brackish environments (10). In the selected high-use ponds, sampling locations were chosen due to visual waterfowl use (molted feathers, birds visually seen, feces left behind, etc.) as well as knowledge of historical trapping sites. In low-use ponds, sampling sites were chosen on similar criteria; although if there was no physical sign of waterfowl, sampling locations were replicated in a similar fashion to those within the high-use ponds (e.g., similar water depth, distance from pond/island edge, and aquatic vegetation).

Sample collection. Samples were collected below the water's surface, midway down the water column, with no surface water allowed in the 10-liter carboy. Measurements of pH, temperature, dissolved oxygen, and conductivity were recorded with the YSI Professional Plus sensor at each of the five locations within an individual wetland pond, at the same depth water samples were taken from. Along with YSI meter readings, other physical environmental variables were also recorded including water depth, soil sample depth and distance from the water's edge, emergent and submergent vegetation, as well as whether or not the area was shaded. We also recorded/estimated the numbers of each bird species observed upon arrival at each pond for sampling. At each of the five locations, a 10-liter water sample was collected according to the lower limit of large volumes considered to be adequate for determining pathogen presence in water (11). A 15 mL surface water sample was collected in a 15mL conical tube from each wetland pond to compare with previous sampling methods. One 15 mL sediment sample was collected in a 15 mL conical tube at each individual sampling site within a wetland to compare the presence and persistence of AIV in sediment to water samples. Sediment samples were collected 1 meter or less from the water's edge, with a nearby correlation to the water sample locations. Sediment samples were obtained at the level of the water table. GPS coordinates for all sampling sites have been recorded, in order to retain accuracy for the sampling of the overwintering and migration period in January/February, as well as future sampling periods. 10-liter water samples were kept on ice and taken back to the lab, where they were stored in a 4°C walk-in fridge, for next-day filtration. Surface water and soil samples were frozen at -80°C to preserve viral integrity prior to RNA extraction. To compare the potential AIV findings from the water and sediment samples, an aimed quota of at least 10 cloacal and oropharyngeal swab samples from live waterfowl was desired to be met from ponds where trapping efforts are being conducted from federal agency partners (USFWS and USGS).

Filtration methods. Conventional tangential flow ultrafiltration separates solutes that differ by tenfold in size through membrane pore size, qualifying this method of filtration as an appropriate approach for AIV detection in larger volumes of water (12). Viral particles were retained by molecular weight cut-offs and concentrated in the retentate while molecules smaller than the filter's pore size flowed through the membrane (13). Prior to filtration, each filter was primed with 500 mL of 0.1NaPP/1L Deionized water. Each 10-liter carboy was filtered using individual Asahi-Kasei Rexeed 25s columns. Each 10-liter carboy was filtered down to a 15 mL retentate to be comparable with the 15 mL unfiltered surface water sample. Once viral particles were caught in the filter membrane, the excess permeate flowed out of the system and into a permeate reservoir, where it was later discarded. Pressure of the filtration system did not exceed 20 psi, and typically ran between 10-15 psi. Upon completion, each filter was eluted with a 500 mL solution of (0.1g NaPP, 10mL 0.01% Tween 80, 10mL 0.001% Antifoam)/1L Deionized water, in order to flush the virus from the membrane into the retentate solutions to be captured. Final samples were immediately placed in the -80°C freezer to preserve viral integrity, until RNA extraction was performed.

PCR and sequencing. RNA from water and swab samples (from wild caught birds) were extracted using the AllPrep PowerViral DNA/RNA (QIAgen). Following extraction (immediately after extraction for water samples), we subjected RNA samples to Influenza A virus whole-segment amplification using multi-segment RT-PCR (14). This procedure uses primers that are complementary to genome segment packaging regions (uni12 and uni13), which are conserved among all influenza A viruses, including

AIV. Thus, this procedure amplifies entire gene segments if they are present in the sample. We conducted gel electrophoresis on select samples to confirm genome segment amplification. Amplicons were barcoded in a second PCR using the Oxford Nanopore PCR Barcoding Kit (Oxford Nanopore Technologies), then multiplexed and prepared for sequencing on R10.4 flow cells (aka Q20+) on the MinION sequencer (Oxford Nanopore Technologies). The MinION sequenced single DNA molecules and allowed for the recovery of entire influenza genome segments (15,16). We reference this method as amplification/sequencing hereafter.

Bioinformatics analyses. Output from the MinION sequencer was analyzed using a custom pipeline that is openly available online. Briefly, raw signal files (.fast5 format) were base-called using Guppy in high accuracy base calling mode (HAC). After quality filtering using Nanofilt (17), reads were demultiplexed (i.e. assigned to a sample) and primers trimmed using Guppy, with both barcodes matching. We used a single brand-new flow cell and included negative and positive controls throughout the sample workflow. NCBI command line BLAST using GNU Parallel (18) was used to search demultiplexed files against all avian influenza whole genome sequences available in the NIAID Influenza Research Database (IRD) (19). Sample metadata from IRD was used to annotate likely subtypes and hosts of AIV sequences detected in each collecting location, based on the closest match in the IRD.

RESULTS

Samples for the pre-migration sampling period were collected in the months of August to mid-September of 2021. In that time, 60 water samples and 50 soil samples were collected from each of the two aforementioned basins. From live birds in the designated Sacramento Basin, 182 oropharyngeal and cloacal swabs were collected as well, in order to compare the AIV ecology and prevalence to that in the environment (Table 1). For designated high-use wetland areas, the amount of waterfowl observed was, on average, multiple hundreds; in low use ponds the number of waterfowl was typically less than 25. Results from the RT-qPCR and sequencing of the collected samples are currently still pending.

DISCUSSION

Waterfowl migrations have been tied to AIV outbreaks in poultry, especially as migrations peak and birds congregate (20). Therefore, understanding the relationship between waterfowl and their habitat with respect to AIV prevalence and viral ecology could provide new insights to commercial poultry farms with respect to AIV risk analysis. The development of targeted surveillance of wild waterfowl populations and their environment remains an active goal for the global poultry industry (21). Next generation applications such as the California and Delmarva Waterfowl Tracker can aid in the monitoring of waterfowl migrations, as well as identify high and low risk areas in the environment through measuring waterfowl density. Here we compare the viral ecology of the environment (i.e. sediment and wetland water) to the AIV found in waterfowl associated with both high- and low-density wetlands. If environmental testing proves successful, a continual surveillance program linking the California Waterfowl Tracker (CWT) to targeted environmental testing could be established, in order to better understand risk to commercial poultry facilities. In one scenario, the combination of the CWT and testing could be used to inform farmers that waterfowl are present or nearby. That information could be used to drive waterfowl away from commercial facilities, as well as heighten biosecurity when a larger threat is present. Other pathways attempting to keep waterfowl off of, and a distance from, poultry facilities have been found to be somewhat successful (such as the utilization of class-III B qualified lasers to scare off aerial birds), but never completely effective (22).

It is clear that on a global scale, there is a significant need for next generation applications like the CWT (20,21,23,24). With systems like this, in cooperation with environmental sampling, as well as sampling from wild birds themselves, the persistence and ecology of AIV in waterfowl populations can be understood, and help reduce the chances of potential outbreaks.

ACKNOWLEDGEMENTS

Sacramento National Wildlife Refuge Complex, Upper Butte Basin Wildlife Area, and Gray Lodge State Wildlife Area staff for the allowance of sample collection

Andrea Mott, Jacob Chappell, and Martin Carriere of USGS-Dixon for the collection of AIV swabs.

UC Davis students that helped in the collection, processing, and filtration of samples.

REFERENCES

1. Hall JS, Grear DA, Krauss S, Seiler JP, Dusek RJ, Nashold SW, Webster RG. Highly pathogenic avian influenza virus H5N2 (clade 2.3.4.4) challenge of mallards age appropriate to the 2015 midwestern

poultry outbreak. *Influenza Other Respir Viruses*. 15:767-777; 2021 Nov.

2. Blagodatski A, Trutneva K, Glazova O, Mityaeva O, Shevkova L, Kegeles E, Onyanov N, Fede K, Maznina A, Khavina E*, et al.* Avian Influenza in Wild Birds and Poultry: Dissemination Pathways, Monitoring Methods, and Virus Ecology. *Pathogens.* 10: 2021 May 20.

3. Stenkamp-Strahm C, Patyk K, McCool-Eye MJ, Fox A, Humphreys J, James A, South D, Magzamen S. Using geospatial methods to measure the risk of environmental persistence of avian influenza virus in South Carolina. *Spat Spatiotemporal Epidemiol*. 34:100342; 2020 Aug.

4. European Food Safety A, European Centre for Disease Prevention CEURLfAI, Adlhoch C, Fusaro A, Gonzales JL, Kuiken T, Marangon S, Niqueux E, Staubach C, Terregino C*,* et al*.* Avian influenza overview May - September 2021. *EFSA J.* 20:e07122; 2022 Jan.

5. Gorsich EE, Webb CT, Merton AA, Hoeting JA, Miller RS, Farnsworth ML, Swafford SR, DeLiberto TJ, Pedersen K, Franklin AB*, et al.* Continental-scale dynamics of avian influenza in U.S. waterfowl are driven by demography, migration, and temperature. *Ecol Appl.* 31:e2245; 2021 Mar.

6. McDuie F, Matchett EL, Prosser DJ, Takekawa JY, Pitesky ME, Lorenz AA, McCuen MM, T OC, Ackerman JT, De La Cruz SEW*, et al.* Pathways for avian influenza virus spread: GPS reveals wild waterfowl in commercial livestock facilities and connectivity with the natural wetland landscape. *Transbound Emerg Dis.* 2022 Jan 2.

7. Acosta S, Kelman T, Feirer S, Matchett E, Smolinsky J, Pitesky M, Buler J. Using the California Waterfowl Tracker to Assess Proximity of Waterfowl to Commercial Poultry in the Central Valley of California. *Avian Dis.* 65:483-492; 2021 Sep.

8. Fleskes JP, Yee JL. Waterfowl Distribution and Abundance During Spring Migration in Southern Oregon and Northeastern California. *Western North American Naturalist*. 67:409-428; 2007.

9. McCuen MM, Pitesky ME, Buler JJ, Acosta S, Wilcox AH, Bond RF, Diaz-Munoz SL. A comparison of amplification methods to detect Avian Influenza viruses in California wetlands targeted via remote sensing of waterfowl. *Transbound Emerg Dis.* 68:98-109; 2021 Jan.

10. Bianchini EA, Bogiatto RJ, Donatello RA, Casazza ML, Ackerman JT, De La Cruz SEW, Cline TD. Host Correlates of Avian Influenza Virus Infection in Wild Waterfowl of the Sacramento Valley, California. *Avian Dis*. 2021 Dec 22.

11. Morales-Morales HA, Vidal G, Olszewski J, Rock CM, Dasgupta D, Oshima KH, Smith GB. Optimization of a reusable hollow-fiber ultrafilter for simultaneous concentration of enteric bacteria, protozoa, and viruses from water. *Appl Environ Microbiol*. 69:4098-102; 2003 Jul.

12. Christy C, Kuriyel R, Bolton G, Seilly A. High-performance tangential flow filtration: a highly selective membrane separation process. *Desalination*. 144:133-136; 2002 10 September 2002.

13. Hill VR, Hahn D, Narayanan J, Cromeans TL, Roberts JM, Amburgey JE. Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants. *Appl Environ Microbiol*. 71:6878-6884; 2005 November 2005.

14. Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, Wentworth DE. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. *J Virol*. 83:10309-13; 2009 Oct.

15. Imai K, Tamura K, Tanigaki T, Takizawa M, Nakayama E, Taniguchi T, Okamoto M, Nishiyama Y, Tarumoto N, Mitsutake K, et al. Whole Genome Sequencing of Influenza A and B Viruses With the MinION Sequencer in the Clinical Setting: A Pilot Study. *Front Microbiol*. 9:2748; 2018.

16. Wang J, Moore NE, Deng YM, Eccles DA, Hall RJ. MinION nanopore sequencing of an influenza genome. *Front Microbiol*. 6:766; 2015.

17. De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics*. 34:2666-2669; 2018 Aug 1.

18. Tange O. Gnu parallel-the command-line power tool. *The USENIX Magazine*. 36(1):42-47; 2011.

19. Zhang Y, Aevermann BD, Anderson TK, Burke DF, Dauphin G, Gu Z, He S, Kumar S, Larsen CN, Lee AJ, et al. Influenza Research Database: An integrated bioinformatics resource for influenza virus research. *Nucleic Acids Res*. 45:D466-D474; 2017 Jan 4.

20. Ferenczi M, Beckmann C, Klaassen M. Rainfall driven and wild-bird mediated avian influenza virus outbreaks in Australian poultry. *BMC Vet Res.* 17:306; 2021 Sep 14.

21. Gobbo F, Fornasiero D, De Marco MA, Zecchin B, Mulatti P, Delogu M, Terregino C. Active Surveillance for Highly Pathogenic Avian Influenza Viruses in Wintering Waterbirds in Northeast Italy, 2020-2021. *Microorganisms*. 9: 2021 Oct 20.

22. Walsh MG, Mor SM, Hossain S. Highly Pathogenic Avian Influenza (H5N1) Landscape Suitability Varies by Wetland Habitats and the Degree of Interface between Wild Waterfowl and Poultry in India. *Viruses*. 12: 2020 Nov 11.

23. Elbers ARW, Gonzales JL. Efficacy of an automated laser for reducing wild bird visits to the free-range area of a poultry farm. *Sci Rep.* 11:12779; 2021 Jun 17.

24. Gulyaeva M, De Marco MA, Kovalenko G, Bortz E, Murashkina T, Yurchenko K, Facchini M, Delogu M, Sobolev I, Gadzhiev A, et al. Biological Properties and Genetic Characterization of Novel Low Pathogenic H7N3 Avian Influenza Viruses Isolated from Mallard Ducks in the Caspian Region, Dagestan, Russia. Microorganisms. 9: 2021 Apr 17.

Figure 1.

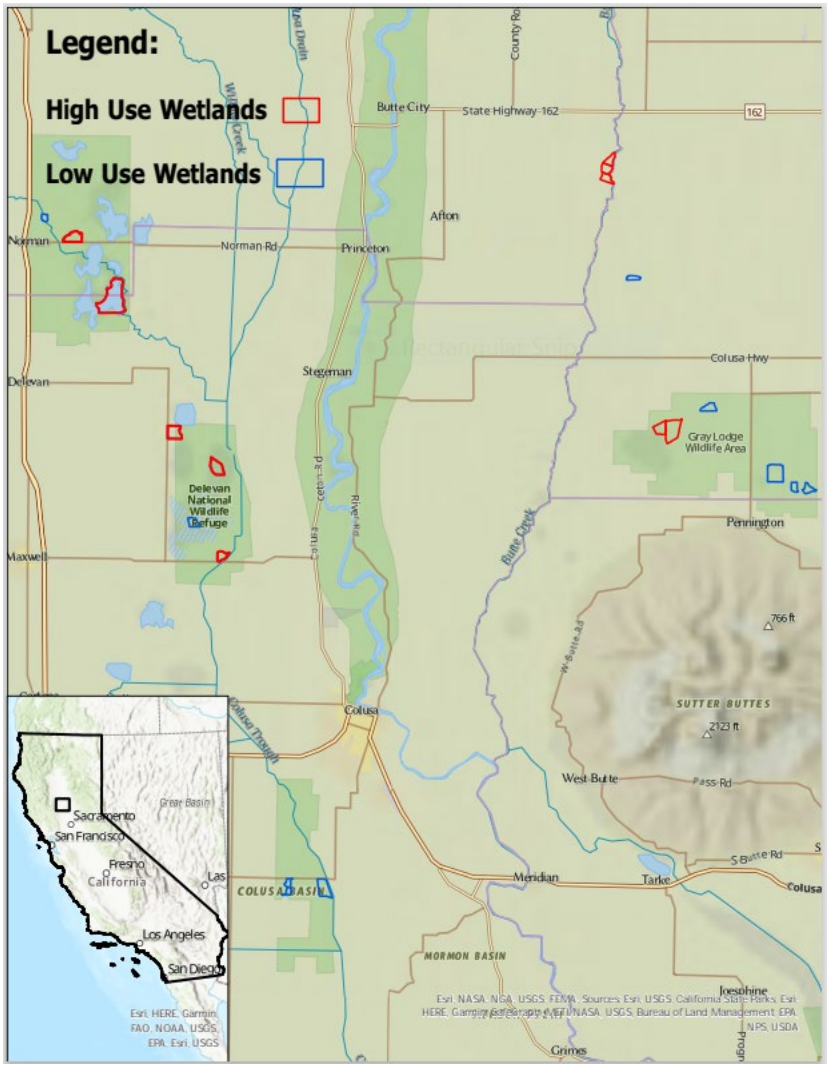


Table 1.

Sampling Period	Basin	Filtered Water Samples	Surface Water Samples	Soil Samples	Avian Swabs
Summer 2021	Sacramento Basin	50	10	50	182
	Butte Basin	50	10	50	--
Winter 2022	Sacramento Basin	50	10	50	80
	Butte Basin	50	10	50	80

ANTIMICROBIAL CHARACTERIZATION OF CHICKEN RIBONUCLEASE ANGIOGENIN 4

J. Rodriguez-Lecompte[A], N. Nazeer[B], S. Uribe-Diaz [A,B], M. Ahmed[B], S. Sharif[C], and J. Reyes[D]

[A]Department of Pathology and Microbiology, Atlantic Veterinary College
[B]Department of Chemistry, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada, C1A 4P3 [C]Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1
[D]Biogenesis Research Group, Faculty of Agricultural Sciences, University of Antioquia, C.P. 050010 Medellin, Colombia

Corresponding author: jrodriguez@upei.ca 550 University Avenue Charlottetown, PE, Canada. C1A 4P3

SUMMARY

Innate intestinal defenses are important for protection against ingested and commensal microbes. Intestinal epithelial cells are the major producers of substances with antimicrobial activity in the intestine. The most abundant and diverse of these are the Defensins. The RNase A super family are also important antipathogenic proteins involved in host-defense mechanisms in vertebrates. We have successfully shown the amplification, cloning, and molecular characterization of a new chicken gene (angiogenin 4), found for the first time in a non-mammalian species, from intestine epithelial and lymphoid cells under nonpathologic conditions. The ability to induce the production of this new class of microbicidal in chickens should enable us to understand the links between innate and acquired immunity in poultry. In this study, chicken (ch) ANG-4 antimicrobial pattern was studies in birds receiving probiotics and organic acids at different period of their life cycle.

INTRODUCTION

Antimicrobial growth promoters (AGP) have been added to poultry feeds in low, sub therapeutic amounts for over five decades (1). The AGP, used as feed additive, demonstrated improvement in growth and feed efficiency in animals (2). The mechanism proposed to explain the AGP-mediated growth enhancement is that the intestinal microflora depresses animal growth; consequently AGP activity is based on its antibiotic properties (3). Nevertheless, lately the use of AGP has been examined for their potential development of antibiotic-resistant human pathogenic bacteria after long continuous use (4). Therefore, it is important to find potential non-pharmacological alternatives to antibiotics with similar or better properties. New alternatives include the use of enteric microflora conditioners such as probiotics, prebiotics, microflora enhancers, enzymes, acidifiers, immunomodulators and herbal products (5).

The demonstration that immune and epithelial cells can discriminate between microbial and bioactive plant species has extended the known mechanisms of action of nutraceuticals and probiotics beyond simple nutrition and/or antimicrobial effects (6). Probiotic strains exert their beneficial effects through a variety of mechanisms that are unique to each strain (7). Angiogenin belongs to the group of proteins called, 'Ribonucleases' with cytotoxic and bacterial properties (8). Interesting in our laboratory we were looking for an avian model (chickens) to characterize the molecular foundations of commensal host-bacterial interactions in the gut, and found that previously uncharacterized chicken ribonuclease is expressed in the intestinal epithelial cell (Losada *et al*., 2020). It is known that gene expression of this novel antibacterial protein is induced by gram negative bacteria such as BT, a predominant member of the gut microflora, revealing a mechanism whereby intestinal commensal bacteria influence gut microbial ecology and shape innate immunity.

MATERIALS AND METHODS

RNA was obtained from intestinal samples to produce cDNA and amplify the full coding chicken Ang4 by PCR. Amplicons were cloned in a pGEM-easy plasmid. His-tagged chAng4 protein was produce subcloning chAng4 in a pET3a plasmid and sequenced. Recombinant product was introduced in a *E. coli* BL-21 expression system. Protein was purified by affinity purification, determined by Bradford protein assay, and endotoxin removed (<1 EU/ug). Protein assessment was done for SDS-PAGE and western blot. Antibodies against purified chAng4 were produced in mouse. Antimicrobial activity was calculated against Gram negative (*S.* Enteritidis) and

Gram-positive (*C. perfringens* and *L. monocytogenes*) intestinal microorganisms, as the amount of CFUs remaining after exposure to increasing concentrations (uM) of purified chAng4.

RESULTS

Amplicon contained a Met codon in place of the signal peptide sequence of Ang4 was obtained. Sequencing of a cDNA clone derived from transcription shown that it encoded chAng4 which is 99% identical to mouse Ang4. It was produced a chicken Ang4 mRNA encoding a 127-residue protein with a predicted amino terminal signal peptide, indicating that it is secreted. The protein length was 127 AA, MW 14784, >90% pure and endotoxin free <1EU/ug. The number of colonies forming units (CFU) of log-phase *Salmonella* Enteritidis declined by 95% after six hours to 16 uM of chAng4. However, *Clostridium perfringens* and *Listeria monocitogenes* 99 and >96% decline in CFU to 2 and 6 uM after at 4 h exposure.

CONCLUSION

We have characterized a new class of chicken endogenous microbicidal protein belong to the RNase family cationic peptides. The results indicate that exist a specific antimicrobial activity inferring selective pressures on the host by differing microbes.

REFERENCES

1. Bywater RJ. Identification and surveillance of antimicrobial resistance dissemination in animal production. Poul Sci 84(4):644-8. 2005.

2. Butaye P., Devriese LA., and Haesebrouck. Antimicrobial growth promoters used in animal feed: effects of less well-known antibiotics on gram-positive bacteria. Clin Microbiol Rev 16(2):175-88 2003.

3. Dudley-Cash WA. A landmark contribution to poultry science--a bioassay for true metabolizable energy in feeding stuffs. Poult Sci 88(4):832-4 2007.

4. Aarestrup FM. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. Basic Clin Pharmacol Toxxicol 96(4):271-81. 2005.

5. Odetallah NH., Ferket PR., Grimes JL., and McNaughton JL. Effect of mannan-endo-1,4-beta-mannosidase on the growth performance of turkeys fed diets containing 44 and 48% crude protein soybean meal. Poult Sci 81(9):1322-31 2002

6. Marco ML., Pavan S., and Kleerebezem M. Towards understanding molecular modes of probiotic action. Curr Opin Biotechnol 17(2):204-10. 2006.

7. Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology 126(6):1620-33. 2004.

8. Cho S., Beintema JJ., and Zhang J. A The ribonuclease A superfamily of mammals and birds: identifying new members and tracing evolutionary histories. Genomics 85(2):208-20. 2005.

9. Losada-Medina D., Yitbarek A., Nazeer N., Uribe-Diaz S., Ahmed M., and Rodriguez-Lecompte JC. Identification, tissue characterization, and innate immune role of Angiogenin-4 expression in young broiler chickens. Poult Sci 99(6):2992-3000. 2020.

LONG TERM PROTECTION OF A DUAL INSERT HVT VECTORED VACCINE (HVT+IBD+ILT) IN LAYER FLOCKS

K. Roza Sutherland

Boehringer Ingelheim Animal Health, Duluth, GA 30096

SUMMARY

Five flocks of commercial layers were followed over the course of their 80-week lifespan for long term monitoring of HVT+IBD+ILT vaccination. Flocks were monitored via serology, PCR, and production metrics for evidence of field challenge or clinical disease.

A new trivalent vectored vaccine (HVT+IBD+ILT) was administered at one day of age to layer flocks in Ohio. Five flocks were selected for long term monitoring and were assessed and followed on a regular basis to assess the serological response and field challenges for IBD and ILT. Serology remained consistently steady among all five test flocks for IBD and was low after maternal antibody waning for ILT, when measured by serology. All PCR performed remained negative for ILT through the current age of the flocks (60 weeks). Production remained at or above breed standard to 60 weeks and will be assessed for the remaining 20 weeks of life as the study concludes. Detailed assessment and discussion of the serologic, PCR, and histopathological testing done will be discussed in the presentation.

ACKNOWLEDGEMENTS

Thank you to Dyke Addis, Dr Jessica Higgins and her team for their assistance and cooperation to complete this work.

REFERENCES

1. Data on file: Boehringer Ingelheim.

2. VARIETIES - HY-LINE NORTH AMERICA (hylinena.com).

3. Bovans_Brown_cs_product_guide_North_America_L8110-2-NA.pdf (hendrix-isa.com).

4. Infectious Laryngotracheitis, pp 142-143. Diseases of Poultry 12th ed.

DETECTION OF *SALMONELLA* IN QUAIL EGGS FOR AMBULATORY SALE IN DISTRICTS OF THE CITY OF LIMA-PERÚ

Magali Salas M.[A], Ana C. Zamora.[B], Nilda Castro A.[C], Nicole Lazo.[D], Aldo Cedrón A.[E], Iván Camargo C.[F], and Carla Bardález C.[G]

[A]ALFA BIOL S.A.C, Director, Laboratory, Lima, Perú
[B] LFA BIOL S.A.C, Analyst. Laboratory of Avian Pathology, Lima, Perú
[C] LFA BIOL S.A.C, Supervisor, Laboratory of Microbiology, Lima, Perú
[D]ALFA BIOL S.A.C, Analyst, Laboratory of Microbiology, Lima, Perú
[E]ALFA BIOL S.A.C, Analyst. Laboratory of Avian Pathology, Lima, Perú
[F] ALFA BIOL S.A.C, Coordinator, Laboratory, Lima, Perú
[G] LFA BIOL S.A.C, Supervisor, Laboratory of Avian Pathology, Lima, Perú

SUMMARY

The aim of this study was to estimate the prevalence of *Salmonella* spp. in quail egg samples destined for human consumption in 21 districts of Metropolitan Lima. One thousand five hundred thirty eggs were analyzed from 51 different seller points of quail eggs, a product that is typically consumed by school-age children, and are frequently seen being sold in ambulatory posts. The eggs were analyzed in a pool of 30 eggs per sample, through the ISO 6579-1:2017/A1:2020 method for *Salmonella* spp. detection for both superficial surface and internal content. There was found one strain from El Agustino, which is the fifth district with the most child and adolescent population in Lima. The strain was isolated from the superficial surface and identified as belonging to the C2 group of *Salmonella* spp. and genotyped as a kentucky strain. The presence of *Salmonella* spp. in El Agustino represents an issue of public health importance.

INTRODUCTION

The egg has structures that make it difficult for microorganisms to enter its interior, among which the shell is the most important, in addition to a lysozyme present in the white, which acts on gram-positive bacteria. The egg shell has numerous pores distributed throughout the surface, through these, gaseous exchange between the environment and the egg (CO2/O) is carried out, however, the environmental fluctuations of temperature and humidity during its storage, as well as the excessive storage time and the degree of contamination present in said environment, can deteriorate this important physical barrier such as the shell and allow the entry of microorganisms that are on the outside of the egg towards the yolk (3).

The activity of selling eggs on an outpatient basis is not well regulated in Peru. It is known that the sales stalls are not formal and therefore cannot be registered or registered by the municipalities, the storage conditions are not adequate, they are found in the environment and this is even more harmful in summer since no sales point sampled used a refrigeration system for adequate conservation, close to 0°C (4), which is important to prolong the time in which the bacteria could cross the shell membrane. Likewise, the storage time has a direct impact on the integrity of the internal and external structures of the egg, due to the loss of water and oxygen related to high temperatures and prolonged storage (5). In this study, the storage time could not be estimated because it depends on consumer demands, which in most of the cases are of low-income university and school age.

Regarding the internal content of the egg, it should not present contamination in the oviposition, as long as the breeder is negative for *Salmonella*; however, the passage of the egg through the cloaca can contaminate the shell with enteric type bacteria, which, under inappropriate conditions mentioned above, can penetrate before the cuticle dries and grow inside the egg as *Salmonella* spp., which is also known to use the transovarian and/or vertical route as the egg passes through the vagina or oviduct. Finally, horizontal transmission by vectors such as animals and even man can be mentioned (6). Being the easiest transmission route to handle, applying a good cleaning and disinfection protocol of the environment and of the vendors that handle the egg (3,6).

MATERIALS AND METHODS

Sample collection and processing. Thirty eggs per spot were collected from markets and ambulatory spots of different districts of Metropolitan Lima. The

samples were transported to ALFA BIOL laboratory in Lima. Where all samples were processed according to the guidelines by International Standard Organization (ISO) 6579-1:2017/A1:2020 to detect *Salmonella* spp., both in egg shell and internal content.

Egg shell processing. Each 30 eggs per sample were mixed with 100 mL of buffered saline solution and rinsed for pre-enrichment. Later, that solution was added BPW and incubated at 37°C for 24 hours.

Egg internal content processing. After the rinse, each egg was opened and homogenized in a glass flask, placed BPW and incubated at 37°C for 24 hours. After 24 hours, both internal and external (shell) samples were added to MKTTn Broth and RVS medium. For the enrichment was used 10 mL of MKTTn Broth with Novobiocin.

Isolation and confirmation of *Salmonella*. After the enrichment, the samples were subcultured by streaking it in XLD and BGA agar, and then incubated at 37°C for 24 hours. Typical colonies of *Salmonella* on XLD agar have a black center and a slightly transparent zone of reddish, and BGA *Salmonella* colonies appear red/pink in color surrounded by a red halo indicating non fermentation of lactose. After that time, suspicious colonies were identified biochemically with TSI / LIA and Urea agar. The typical *Salmonella* culture show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and formation of hydrogen sulfide (blackening of the agar) in TSI agar. For Lysine agar Turbidity and a purple color after incubation indicate a positive reaction and a result negative to hydrolyze urea.

Serotyping. The presumptive pure colonies are tested eliminating auto-agglutination strains. A colony identified as *Salmonella* spp. was impregnated in FTA cards and sent to the Georgia Poultry Laboratory Network for the serotyping service.

RESULTS

A total of 1530 fresh quail eggs were collected from 51 retail markets and ambulatory spots of 21 districts of Metropolitan Lima, making a total of 51 samples, during the period from October to November 2021, spring season in Peru (Table 1). The present study showed presence in one (1.96%) sample analyzed from the egg shell (Table 2). The serotyping of the isolated strain showed coincidence with *Salmonella* Kentucky.

DISCUSSION

In this study, eggs for human consumption marketed on an outpatient basis in the city of Lima were analyzed, in such a way that their origin is unknown.

The aim of the present study was to detect the presence of *Salmonella* spp. in the internal part of raw quail eggs, collected from ambulatory posts of 21 districts of Metropolitan Lima, to detect the presence of contaminated eggs in said spending points. As cited by Gast and Saed, *Salmonella* can colonize the interior of the egg just before the incorporation and effect of the surface protein of the shell on the egg, since this forms a barrier that prevents the invasion of bacteria, which allows this microorganism to colonize and survive in the internal contents of the egg (1,2).

After analyzing all the samples, the present research detected *Salmonella* spp. in an analyzed sample (egg shell), belonging to the El Agustino district. This proves that it is possible to find this bacterium in this type of product, meaning a high risk of enteric disease for consumers, if care is not taken in the cooking method of the product.

In our country, this is the first work carried out in quail, research work has been carried out regarding *Salmonella* spp. in pink chicken eggs in intermediate markets of Metropolitan Lima (7), where 64 egg samples were analyzed, but did not obtain positive results, which could indicate that this form of sale is more regulated than the ambulatory one. In the case of chicken eggs, the regulation in Peru indicates a labeling of the batch and expiration date of the egg, appropriate storage conditions and knowledge of the origin of the product at the point of sale. Another work on chicken eggs was done by Levano G. and López C, who reported a study in which 680 eggs from four poultry farms and some markets in Lima were analyzed, in which 21 strains of *Salmonella* were obtained: *enterica* of the Djugu and Mbandaka serotypes (8).

Due to the desire to investigate more about this product, which is the food alternative for many families, and also a source of income, since in Peru the raising of these birds it is familiar and is carried out in a low-tech and less expensive way. Quail are birds that are relatively resistant to various infectious diseases due to their high content of lysosomes (9); they reach sexual maturity at seven weeks of age instead of the 20-24 weeks required for a hen to start mating lay eggs. Furthermore, quail have a high egg laying rate (280-300 eggs per year), and immunization does not result in reduced egg production (9).

However, despite all these breeding advantages and the internal composition of the eggs of these birds, this study had a positive result for *Salmonella* Kentucky in the samples analyzed, which differs from what has been held in theory about the resistance of these birds to *Salmonella* (10). In other words, the amount of lysosomes that the quail egg contains as a barrier to bacterial infection could work for *Salmonella* Typhimurium and *Salmonella* Enteritidis.

With this study it could be shown that it is not effective for other types of *Salmonella* such as Kentucky.

These results suggest periodically carrying out more analyzes of this type, extending the sample size and also analyze the hands of the people who sell these eggs, as possible sources of contamination for humans, thus create records and apply statistics to the epidemiology of bacteria in our country, to help improve its control and dissemination to the highest-risk consumers.

ACKNOWLEDGMENTS

The authors would like to thank the assistants and commercial and research team working in ALFA BIOL for their help in the present study.

REFERENCES

1. Gast RK, Beard CW. Production of Salmonella Enteritidis contaminated eggs by experimentally infected hens. Avian Dis. 34(2):438-46; 1990.

2. Saaed A, Gast R, Potter M, Wall P. Salmonella enterica serovar Enteritidis in Humans and Animals: Epidemiology, Pathogenesis, and Control. Ames, Iowa: Iowa State University Press. (Estados Unidos). 443 p; 1999.

3. Jay, James M.. Microbiologia de Alimentos. 6th ed. Porto Alegre: Artmed, 2005.

4. Fica A, M Alexandre, S Prat, A Fernández, J Fernández, I Heitmann. Cambios epidemiológicos de las salmonelosis en Chile. Desde Salmonella typhi a Salmonella enteritidis. Rev Chil Infectol, 18, 85-93; 2001.

5. Lublin A, Sela S. The impact of temperature during the storage of table eggs on the viability of Salmonella enterica serovars Enteritidis and Virchow in the eggs. Poultry Science; 87(11):2208-14; 2008.

6. De Buck J, Pasmans F, Van Immerseel F, Ducatelle R. Tubular glands of the isthmus are the predominant colonization site of Salmonella Enteritidis in the upper oviduct of laying hens. Poultry Science. 83(3):352-8; 2004.

7. Cerron B, Presencia de Salmonella spp. en la parte interna de huevos rosados para el consumo humano en mercados intermedios de Lima Metropolitana y Callao registrados por el Instituto Metropolitano de Planificación [thesis]. [Lima] Universidad Privada Cayetano Heredia, 2019.

8. Levano G, López C. Evaluación de la presencia de Salmonella en huevos frescos utilizando el medio Xilisa-Lisina-Tergitol 4 (XLT4). Cienc Invest. 2001; IV(1): 50- 56; 2001.

9. Valle S, et al. Manual Crianza y Manejo de codornices. Managua, Nicaragua 2015.

10. Atefeh E, Bahman A, Elhamsadat H, Maryam S. Producción, purificación y evaluación de inmunoglobulina Y de codorniz contra Salmonella typhimurium y Salmonella enteritidis; 2019.

Table 1. Occurrence of isolation of *Salmonella* spp. in the districts sampled.

	Isolation		
District	No (n)	Yes (n)	Total
San Juan de Lurigancho	3	0	3
San Martín de Porres	5	0	5
San Juan de Miraflores	3	0	3
Lince	3	0	3
El Agustino	9	1	10
Comas	1	0	1
San Bartolo	1	0	1
San Borja	1	0	1
Surquillo	3	0	3
La Victora	4	0	4
Pueblo Libre	1	0	1
El Rimac	3	0	3
Centro de Lima	1	0	1
Magadalena del Mar	1	0	1
Santa Anita	4	0	4
Punta Hermosa	1	0	1
Lurin	1	0	1
El Callao	2	0	2
Villa El Salvador	1	0	1
Villa María del Triunfo	1	0	1
Los Olivos	1	0	1
Total	50	1	51

Table 2. Occurrence *Salmonella* spp. on egg shell and internal content.

	Quail eggs	
Type of sample	Number	% Positive
Egg shell	1	1.96
Internal content	0	0.00
Total (n=51)	1	1.96

USING MULTI-MODAL FEED ADDITIVES TO HELP MANAGE COCCIDIA CHALLENGES AND THEIR SEQUELAE IN BROILER PRODUCTION SYSTEMS

D. Sandu[A]

[A]Alltech Inc., 3031 Catnip Hill Rd., Nicholasville, KY, 40356

SUMMARY

Coccidiosis continues to be one of the major diseases of importance worldwide. Primarily, coccidiosis is thought as a disease of economic impact due to the losses associated with production. As the demand for poultry meat that is raised without antibiotics, including ionophores, continues to increase, major challenges in controlling coccidiosis have left the industry with limited treatment and prevention choices. Another concern that stems from coccidiosis is its sequelae. A rise in secondary bacterial infections and gastrointestinal imbalance have become more concerning. Therefore, as the poultry industry, particularly the broiler sector, continues to look for treatment and preventative solutions, emphasis in integration of feed additives with multi-modal applications has been a major area of exploration. Another area of struggle for the industry has been how to consistently integrate a holistic approach while maintaining sustainability, productivity and animal welfare while using these products.

In this article we discuss how the use of some feed additives with different modes of action may be of used in managing coccidiosis challenges and other secondary bacterial infections.

COCCIDIOSIS IN POULTRY AND SEQUELAE

Coccidiosis is caused by intestinal infection by the protozoan parasite in the genus *Eimeria*. In commercial poultry production, the signs of coccidiosis may not immediately noticeable as subclinical coccidiosis tends to occur frequently. Coccidiosis can result in intestinal lesions, diarrhea, enteritis, morbidity, and mortality, while subclinical coccidiosis may only appear as varying degrees of decreases in performance parameters such as feed conversion and reduced weight gains (1). This "milder" clinical sign often makes diagnosing coccidia difficult and prevents the efficient and timely use of control strategies (2).

During the lifecycle of coccidia, replication and oocyte production occurs in the intestines which enters the host through the ingestion of sporulated oocysts from the contaminated environment (3, 4). Depending on the *Eimeria* species (spp.), different locations of the intestine and the ceca can be affected. During the endogenous phase of coccidia cycling, which includes: colonization, growth, and reproduction, most of the damage occurs in the small intestine and ceca (5). Cycling causes disruption of the intestinal mucosa, immunity, and the balance of the intestinal microbiota (6). Externally the birds may display ruffled feathers, vent pasting from diarrhea, increased foot pad dermatitis from increased wet litter, and depending on the type of coccidia, increased morbidity and mortality (7). The house litter environment may be another useful indicator as evidence of diarrhea, increased mucus in feces, increased undigested feed and increased ammonia odor can be appreciated (7).

While the long-term effects of coccidiosis are mostly economic in nature, there are some sequelae which could indirectly associated to coccidia and cycling events. Secondary bacterial infections such as necrotic enteritis, co-infection with other protozoal infections, failure to achieve full immunity from vaccines and increases in pathogenic bacteria of food safety concern have been implicated to coccidiosis as one of the causative agents (8, 9). This is attributed to the incurred damage to gut mechanical barriers which increases intestinal permeability, interactions with the chemical barriers particularly mucus secretion, compromising of the immune system's cellular and humoral components (8, 10). The effects coccidiosis on the gut microbiota can be observed throughout the life of the flock and can carry over into the food chain particularly regarding increases of infection with *Salmonella* and *Campylobacter* (9, 11, 12). The lingering sequelae of coccidiosis can also contribute to the overall flock's morbidity and mortality hence compromising productivity as well as increasing animal welfare concerns.

COCCIDIA CONTROL IN COMMERCIAL POULTRY

In the past, synthetic anticoccidial drugs and antibiotics, including ionophores, were primarily used

prophylactically in commercial poultry rearing practices to control coccidiosis. However, concerns of resistance and consumer trend preferences have shifted the use of these strategies to mitigate coccidiosis (13). Within the last decade, the use of attenuated coccidia vaccines, ionophore and synthetics in addition to usage of these chemoprophylactics in rotation and bio-shuttle programs have helped with control coccidia, particularly in conventional poultry (1, 4). Additionally, stronger implementation of biosecurity, cleaning, and disinfection and longer down-time between flocks has helped decrease challenge as well as introduction of resistant *Eimeria* spp. (14). Vaccination as means of prevention is particularly popular in the US and it has shown some advantages because it doesn't induce drug resistance and could help decrease resistant coccidia on farm. Vaccine use also allows for early harvest without constraints of drug withdrawal periods (4, 15).

In poultry rearing practices that have label claims such as antibiotic free, no antibiotics ever and organic poultry, limitations on product use has challenged the industry to mitigate coccidiosis and therefore, the use of natural alternatives with coccidiostatic effects and modulation of the bird's natural ability to fight off infections has been a greater focus (4, 6).

FEED ADDITIVE ALTERNATIVES WITH MULTI-MODAL APPLICATIONS AGAINST COCCIDIOSIS

Feed additives are products that represent different classes of molecules, compounds, and beneficial organisms which enhance nutrition, feed preservation, help digestion, metabolism and improve animal health (16). In poultry, some commonly used natural feed additives can include single or combination preparations of acidifiers, antibiotics, prebiotics, probiotics, postbiotics, plant derivatives, extracts, immunostimulants, flavoring agents, antioxidants, vitamins, minerals, enzymes, amongst others. As the shift in the industry has been less favorable towards the use of antibiotics as growth promoting and anticoccidial agents, naturally derived materials that help decrease the effects of coccidiosis have been increasing in popularity (16).

Saponins, a secondary metabolite derived from plants such as those from *Yucca schidigera* are well known to interact with the development of coccidia parasites (17, 18). Saponins can cause protozoal cell membrane rupture and lysis leading the death of the parasite through irreversible complex formation with the cholesterol present on the parasite cell membrane (19, 20). In addition to antiprotozoal activity, saponins help promote digestion and absorption of nutrients and exert nonspecific immunomodulatory effects.

Another natural feed additive with antioxidant, anticoccidial, antimicrobial and antifungal effects are essential oils (21). Although the mode of action of these is not fully understood, they have been appealing fit for the poultry industry. Their main activity is due to its composition of phenols, carvacrol and thymol (22). These components of essential oils can interfere with bacterial energy metabolism, bacterial membrane stability, bacterial membrane protein synthesis and activity. Their effect highly depends on the membrane arrangement and cell wall structure of the microorganism (21). Although the effects are variable between different plant essential oil extracts, some commonly used ones with anticoccidial and antibacterial effects are those derived from oregano plants mainly due to its polyphenol composition. Therefore, not all plants in the same species or subspecies will exhibit the same composition and may not display the same outcome against coccidia or bacteria which leads to effectivity inconsistencies (22).

Prebiotics, such as mannan oligosaccharides (MOS) derived from yeast cell wall *Saccharomyces cerevisiae* are well described for the benefits to the hosts through various functionalities. They can improve feed efficiency as well as influence the gastrointestinal microbiota (23-25). Mannan-rich fractions (MRF's) supplemented in broiler diets are capable of binding to fimbriae on pathogenic bacteria, which prevents colonization to the gut wall and multiplication (26, 27). They are also involved in interactions with the immune system and can enhance immune activity, particularly at the local gastrointestinal tract level (23). Their capability as an intestinal immunomodulator is also described to work in conjunction with coccidia vaccine programs (28).

Another category of feed additives with beneficial properties during coccidia challenges are antioxidants. Coccidia cycling has been involved in imbalance to the antioxidant status of birds. Antioxidants can provide cellular protection against oxidative stress and reduce the severity of coccidia infection (29). Selenium and zinc are key trace minerals that can be incorporated as feed additives. Selenium is essential in the making of selenoproteins which are involved in processes such as cellular maintenance, hormone metabolism, immune responses, and oxidative and calcium homeostasis. Zinc also plays a major role in healing and the support of the immune system, DNA and protein formation and cellular growth (29, 30). During coccidiosis, there are reduced absorption and a decrease in levels of some vitamins and minerals with antioxidant capabilities such as selenium and zinc (31). Decreased bioavailability of these nutrients can lead to increases cellular damage, increases production of lipid

peroxidation products, and decrease the amounts of enzymes involved in the breakdown of oxygen molecules in the cells (30, 32). Organic selenium and organic zinc supplementation in feeds has shown to be more bioavailable than traditionally used inorganic compounds and is also considered to be less toxic when handled or incorporated into the environment (32). Supplementation of selenium and zinc in diets has shown to help immunization of chickens against coccidiosis and improve performance through a reduction in mortality and increased body weight gain (29, 31).

Alone, these feed additives exert different benefits over coccidia, the immune system of the host, the gastrointestinal functionality and repair, and the microbiota of the bird's digestive tract. Although there are many other feed additives with similar effects, saponins, essential oils, prebiotics and organic selenium can be found in combination. As a combination product, they can potentiate each other as well as have practical applications through ease of use. This natural blend of feed additives has been shown to help prevent coccidiosis, reduce lesion scores, improve performance parameters such feed conversion and body weights (33).

IMPACT OF COCCIDIOSIS ON PRODUCTIVITY, ANIMAL WELFARE AND SUSTAINABILITY: A DIFFERENT KIND OF SEQUELAE

The importance of coccidiosis as a disease that affects economic productivity, overall output of meat produced, animal welfare and sustainability has been increasing. Economically, coccidiosis continues to be one of the top diseases of concern particularly in the broiler industry. It is estimated that the loss is due to coccidia is in excess of $3 billion globally (34). In recent re-calculations of the cost of coccidiosis in chickens, in the US alone, it is estimated that using prices from 2016 the total cost of coccidiosis was around $1,574.54 million (34). As feed ingredients and other commodities continue to increase in price, the cost of production is expected to be higher going forward. Therefore, it will be key to maintain productivity and decrease losses during coccidia challenges.

In agriculture, the term sustainability is debated and has been used to describe the notion of benign practices or stability of all involved facets of agriculture and the interaction with society (35). A commonly used definition for the concept of animal sustainability and agriculture was given by Douglas in 1984 where there are three dimensions which include stewardship, food sufficiency, and community (36). This is consistent with other more recent proposed definitions where there are different pillars including ecological, social, and economic aspects that explain achievable goals for sustainability (35, 37). The impact of coccidiosis on sustainability tends gravitate towards affecting the economic pillar directly; however, it also affects the social pillar component under acceptable agricultural practices which includes animal welfare and the environmental pillar which includes farming practices and emission indicators (37). Under the economic sustainability concept, coccidiosis directly affects feed conversion ratios, company or farm revenue and ultimately cost of production. This is mainly due to the performance losses associated with the disease which directly affect profitability. Farms that may be continuously afflicted by uncontrolled coccidiosis may not be able to reach the full potential of their flock having reduced yield outputs and increased cost of production which consequently is not sustainable. Regarding the social pillar, animal welfare has been of great focus in the poultry industry. It requires identification and correction of the problems that would compromise well-being of the animal. During coccidiosis, hindered nutrition and secondary bacterial infections that arise from the disease can compromise the bird's health and directly and indirectly affect the welfare of the bird. Therefore, implementation of programs and appropriate interventions will be needed to decrease the impact of coccidiosis with relation to animal welfare and sustainability.

CONCLUSION

As the shift in the industry continues toward antibiotic free production, it will be key to evaluate the role and the opportunities that natural feed additives provide to poultry. Variability on disease control of coccidia challenge and its sequelae have become more evident. Therefore, efficacious alternative natural feed additives with a variety of modalities may decrease the negative impact of coccidiosis while maintaining the approval of the consumers. Combination products with coccidiosis management benefits may be ideal to compliment or improve current control strategies that target improving overall bird's health, promote the balance of the gastrointestinal microbiota, increase feed efficiency and livability which would ultimately help improve sustainability, welfare, and productivity.

REFERENCES

1. Dalloul, R. A., and H. S. Lillehoj. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev Vaccines 5:143-163. 2006.

2. Blake, D. P., J. Knox, B. Dehaeck, B. Huntington, T. Rathinam, V. Ravipati, S. Ayoade, W. Gilbert, A. O. Adebambo, I. D. Jatau, M. Raman, D. Parker, J. Rushton, and F. M. Tomley. Re-calculating the cost of coccidiosis in chickens. Vet Res 51:115. 2020.

3. Reyna, P. S., L. R. McDougald, and G. F. Mathis. Survival of coccidia in poultry litter and reservoirs of infection. Avian Dis 27:464-473. 1983.

4. Tewari, A. K., and B. R. Maharana. Control of poultry coccidiosis: changing trends. J Parasit Dis 35:10-17. 2011.

5. Mesa-Pineda, C., J. L. Navarro-Ruíz, S. López-Osorio, J. J. Chaparro-Gutiérrez, and L. M. Gómez-Osorio. Chicken coccidiosis: from the parasite lifecycle to control of the disease. Front Vet Sci 8. 2021.

6. Lu, C., Y. Yan, F. Jian, and C. Ning. Coccidia-microbiota interactions and their effects on the host. Front Cell Infect Microbiol 11:751481-751481. 2021.

7. Dunlop, M. W., A. F. Moss, P. J. Groves, S. J. Wilkinson, R. M. Stuetz, and P. H. Selle. The multidimensional causal factors of 'wet litter' in chicken-meat production. Sci Total Environ 562:766-776. 2016.

8. Latorre, J. D., B. Adhikari, S. H. Park, K. D. Teague, L. E. Graham, B. D. Mahaffey, M. F. A. Baxter, X. Hernandez-Velasco, Y. M. Kwon, S. C. Ricke, L. R. Bielke, B. M. Hargis, and G. Tellez. Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens. Front Vet Sci 5:199-199. 2018.

9. Takimoto, H., E. Baba, T. Fukata, and A. Arakawa. Effects of infection of Eimeria tenella, E. acervulina, and E. maxima upon Salmonella typhimurium infection in chickens. Poult Sci 63:478-484. 1984.

10. Latorre, J. D., B. Adhikari, S. H. Park, K. D. Teague, L. E. Graham, B. D. Mahaffey, M. F. A. Baxter, X. Hernandez-Velasco, Y. M. Kwon, S. C. Ricke, L. R. Bielke, B. M. Hargis, and G. Tellez. Evaluation of the Epithelial Barrier Function and Ileal Microbiome in an Established Necrotic Enteritis Challenge Model in Broiler Chickens. Front Vet Sci 5. 2018.

11. Rimet, C. S., J. J. Maurer, R. D. Berghaus, B. J. Jordan, L. H. A. da Silva, L. J. Stabler, K. K. Johnson, L. R. Tensa, K. M. Segovia, and M. S. França. The contribution of Eimeria coinfection and intestinal inflammation to cecal colonization and systemic spread of Salmonella typhimurium deficient in tetrathionate reductase or type III secretion systems Salmonella pathogenicity island 1 or 2. Avian Dis 63:559-567. 2019.

12. Macdonald, S. E., P. M. van Diemen, H. Martineau, M. P. Stevens, F. M. Tomley, R. A. Stabler, and D. P. Blake. Impact of Eimeria tenella Coinfection on Campylobacter jejuni Colonization of the Chicken. Infect Immun 87. 2019.

13. Sundar, S. B., T. Harikrishnan, B. R. Latha, G. Sarath, and C. T. S. Kumar. Anticoccidial drug resistance in chicken coccidiosis and promising solutions: A review. J Entomol Zoo Stud 5:1526-1529. 2017.

14. Peek, H. W., and W. J. M. Landman. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. Vet Q 31:143-161. 2011.

15. Chapman, H. D., and T. K. Jeffers. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. Int J Parasitol Drugs Drug Resist 4:214-217. 2014.

16. Alagawany, M., and M. E. Abd El-Hack. *Natural feed additives used in the poultry Industry*. Singapore: Bentham Science Publishers; 2020.

17. Alfaro, D. M., A. V. F. Silva, S. A. Borges, F. A. Maiorka, S. Vargas, and E. Santin. Use of Yucca schidigera extract in broiler diets and its effects on performance results obtained with different coccidiosis control methods. J of Applied Poult Res 16:248-254. 2007.

18. Scheurer, W., P. Spring, and L. Maertens. Effect of 3 dietary phytogenic products on production performance and coccidiosis in challenged broiler chickens. J Appl Poult Res 22:591-599. 2013.

19. Ramos-Morales, E., G. de la Fuente, S. Duval, C. Wehrli, M. Bouillon, M. Lahmann, D. Preskett, R. Braganca, and C. J. Newbold. Antiprotozoal effect of saponins in the rumen can be enhanced by chemical modifications in their structure. Front Microbiol 8:399-399. 2017.

20. Francis, G., Z. Kerem, H. P. Makkar, and K. Becker. The biological action of saponins in animal systems: a review. Br J Nutr 88:587-605. 2002.

21. Kalemba, D., and A. Kunicka. Antibacterial and antifungal properties of essential oils. Curr Med Chem 10:813-829. 2003.

22. Abd El-Hack, M., M. El-Saadony, N. Yehia, A. Khafaga, M. Farag, M. Alagawany, and M. Abd. Beneficial impacts of essential oils on poultry health and production. In: Alagawany M., and M.E. Abd El-Hack, editors. *Natural feed additives used in the poultry industry*. Singapore: Bentham Science Publishers. p 201-224; 2021.

23. Spring, P., C. Wenk, A. Connolly, and A. Kiers. A review of 733 published trials on Bio-Mos®, a mannan oligosaccharide, and Actigen®, a second generation mannose rich fraction, on farm and companion animals. J Appl Anim Nutr 3:e8. 2015.

24. Corrigan, A., K. Horgan, N. Clipson, and R. A. Murphy. Effect of dietary prebiotic (mannan oligosaccharide) supplementation on the caecal bacterial community structure of turkeys. Microb Ecol 64:826-836. 2012.

25. Baurhoo, B., P. R. Ferket, and X. Zhao. Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. Poult Sci 88:2262-2272. 2009.

26. Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. Poult Sci 79:205-211. 2000.

27. Murphy, R. Microfloral rehabilitation: normalisation of gut function. Int Anim Health J 4. 2017.

28. Nollet, L., G. Huyghebaert, and P. Spring. Effect of dietary mannan oligosaccharide (Bio-Mos) on live performance of broiler chickens given an anticoccidial vaccine (Paracox) followed by a mild coccidial challenge. J Appl Poult Res 16:397-403. 2007.

29. Abbas, R., Z. Iqbal, M. Mansoor, Z. Sindhu, M. Zia, and J. Khan. Role of natural antioxidants for the control of coccidiosis in poultry. Pak Vet J 33:401-407. 2013.

30. Bray, T. M., and W. J. Bettger. The physiological role of zinc as an antioxidant. Free Radic Biol Med 8:281-291. 1990.

31. Bortoluzzi, C., B. Lumpkins, G. F. Mathis, M. França, W. D. King, D. E. Graugnard, K. A. Dawson, and T. J. Applegate. Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and Clostridium perfringens. Poult Sci 98:2211-2219. 2019.

32. Shini, S., A. Sultan, and W. L. Bryden. Selenium biochemistry and bioavailability: implications for animal agriculture. Agric 5:1277-1288. 2015.

33. Duffy, C. F., G. F. Mathis, and R. F. Power. Effects of Natustat™ supplementation on performance, feed efficiency and intestinal lesion scores in broiler chickens challenged with Eimeria acervulina, Eimeria maxima and Eimeria tenella. Vet Parasit 130:185-190. 2005.

34. Williams, R. B. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. Int J Parasit 29:1209-1229. 1999.

35. Thompson, P. Agricultural sustainability: What it is and what it is not. Int J Agric Sustain 5:5-16. 2007.

36. Douglass, G. K. The Meanings of Agricultural Sustainability. Westview Press, Boulder CO. 1984.

37. Yunlong, C., and B. Smit. Sustainability in agriculture: a general review. Agric, Ecosys & Environ 49:299-307. 1994.

LONG-TERM STORAGE OF AVIAN ORIGIN PRIMARY CELLS FOR CONTINUED AVAILABILITY IN VIRUS RESEARCH AND DIAGNOSTIC LABORATORIES

G. Sarma, A. Jones*, M. Stubbs, and J. Diaz

Hygieia Biological Laboratories, Woodland, CA 95776
* Presenting author

SUMMARY

Chicken embryo fibroblast (CEF) cells prepared from 10-day old specific pathogen free (SPF) chicken embryos, chicken liver (CL) cells prepared from 16 to 19 day old embryos and chicken kidney (CK) cells prepared from one to three-week old SPF chicks were tested for their viability and growth potential after storing the cells in liquid nitrogen for varying periods up to four years. Both CEF and CK cells could be stored successfully up to four years. The stored cells maintained its viability and growth potential as evidenced from the 100% confluent monolayer formation within two to three days of seeding depending on the seeding density of the cells. The cryopreserved cells also formed confluent monolayers when passaged serially up to 18 passages. From 18 passage onwards the cells started to show morphological changes and slow growth rate. The CL cells, however, could not be cryopreserved following the current methods used in this study. The cells became nonviable upon storage. A method for cryopreservation of avian origin primary cells is described.

INTRODUCTION

Primary chicken embryo fibroblast, chicken kidney and chicken liver cells are widely used for avian as well as mammalian virus research all over the world (1-7, 9-11, 13-15, 17). These cells are anchorage dependent and unlike established cell lines, they cannot be maintained for a prolonged period by giving serial passages. Therefore, when needed, these cells are prepared fresh from chicken embryos (1, 3-10, 12, 16)) or from young chicks (7). Specific pathogen free embryonated chicken eggs are typically used for preparing the primary CEF cells. For preparation of CK and CL cells, either 16 to 19 days old embryonated eggs (2,11,15) or 1- to 3-week-old young chicks (7,13) are used depending on the need and availability. Preparation of the primary cells is time consuming, expensive and needs an experienced trained person. Availability of a pre-prepared and validated cryopreserved cell lot would be much more convenient, time saving as well as cost effective. In this study, we tried to establish a readily available cell source by cryopreserving the cells in liquid nitrogen (-196°C).

MATERIALS AND METHODS

Preparation of primary CEF cells. The primary CEF cells were prepared as described elsewhere (8). Briefly, 10-day old SPF embryonated eggs were prepared by sanitizing the eggshell surface with 70% alcohol or other equivalent sanitizers. Using sterile forceps, the egg shell covering the air sac in the wider end of the egg was removed. The embryo was pulled out carefully and washed a couple of times using Hanks balanced salt solution (Sigma-Aldrich Company, Catalog No. H9394) containing 100 units/ mL of penicillin and 30 µg /mL of streptomycin as bacteriostatic agents. The embryos were then finely minced using a sterile scissor or other equivalent devices. The minced tissues were trypsinized using 0.25% trypsin solution (Gibco Laboratories, Cat. No. 15050065) under constant stirring or shaking in a 37°C incubator. The trypsinized cells were pelleted down by centrifugation at 1500 rpm for 15 minutes. Pelleted cells were resuspended in cell growth medium (M199, Gibco Laboratories, Cat. No. 11150059) containing 5 -10% fetal bovine serum (FBS) and antibiotics as bacteriostatic agents. The percentage cell viability was determined by trypan blue dye exclusion test.

The number of viable cells in the suspension was counted using a bright-line Hemocytometer (Hausser Scientific, Cat. No. 1483). The cells so prepared were either used fresh to prepare cell monolayers in the cell culture flasks, plates, roller bottles or stored frozen in liquid nitrogen for future use. For seeding the liquid nitrogen stored or freshly prepared cells to make a cell monolayer a cell seeding density of 0.1 to 1.0 million viable cells/mL was used. The cells in the subsequent passages were trypsinized using 0.25% trypsin – EDTA solution (Sigma-Aldrich Company Cat. No. T4049).

Preparation of primary CK cells. The primary CK cells were prepared by using 1- 3-week-old SPF chicks. The kidney tissues were aseptically harvested and processed to prepare the cells. The procedure of preparing the cells were essentially the same as the CEF cells with some exceptions (7). Dulbecco's Modified Eagles Medium (DMEM, Gibco, Cat. No. 10566024) supplemented with 10% FBS and penicillin and streptomycin as bacteriostatic agents was used to grow the freshly prepared or liquid nitrogen stored CK cells in monolayer cultures. For subsequent passages of the kidney cell monolayers 0.25% trypsin – EDTA was used.

Preparation of primary CL cells. For preparation of the primary CL cells, 16 to 19 days old embryonated eggs were used. Liver tissues were carefully harvested from the embryos and processed to prepare the primary hepatocytes. The cells were prepared following the same procedure as already described for the CK cells. DMEM supplemented with 10% FBS and penicillin and streptomycin as bacteriostatic agents was used to grow the freshly prepared or liquid nitrogen stored CL cells in monolayer cultures.

Cryopreservation and revival of stored cells. For storing in liquid nitrogen, the primary cells were resuspended in cell freezing medium containing basal medium, dimethyl sulfoxide (DMSO) as a cryoprotectant, FBS along with penicillin and streptomycin as bacteriostatic agents. The resuspended cells were aliquoted in sterile cryovials (1.0 mL/vial) and subjected to slow freezing for three to six hours before transferring them to liquid nitrogen. The stored cells were revived from liquid nitrogen at various time intervals to evaluate its viability and growth kinetics. In order to revive cells from liquid nitrogen, the desired number of vials were taken out and thawed rapidly at 35 to 37^{o}C. The thawed cells were resuspended in cell growth medium supplemented with FBS and penicillin and streptomycin at a final concentration of 100 units/mL and 30 µg/mL respectively. The cells were then seeded into the appropriate size cell culture vessels (flasks or plates) and incubated in a humidified CO_2 incubator. The incubator temperature was maintained at 37 ^{o} C. Incubation continued until the cells formed a confluent monolayer. Seeding density and viable cell counts were determined prior to transferring the culture vessels to the incubators. Cell yield from the incubated cultures was also determined at the time of harvest.

RESULTS AND DISCUSSION

Primary CEF cells. Five lots of primary CEF cells were prepared and stored frozen in liquid nitrogen. The cell viability prior to storage was 100% for each lot of cells. There was an initial drop of cell viability in the first 3 to 4 weeks of storage. The cell viability remained more or less stable thereafter. Cells were successfully recovered from liquid nitrogen for over 4 years (maximum period tested until now). The revived cells formed 100% confluent monolayers within 48 hours depending on seeding density.

The cell monolayers prepared from the cryopreserved cells could be trypsinized and passaged successfully up to 21 passages. There was no grossly detectable change in growth characteristics or cell morphology when the cells were passaged up to 18 passages. However, from passage 19 the cells started showing changes in morphology and growth rate also slowed down forming only 50% confluent monolayer after seven days of incubation.

Primary CK cells. Two lots of primary CK cells were prepared and cryopreserved successfully in liquid nitrogen. The stored cells maintained its viability and growth potential during storage. The cells revived periodically up to four years of storage formed 100% confluent monolayers within 72 hours of seeding.

Primary CL cells. Two lots of primary cells were prepared for cryopreservation. The freshly prepared cells formed 100% confluent monolayer in two to three days depending on cell seeding density. However, when these cells were stored frozen in liquid nitrogen, the cells became non-viable indicating that the method of preparation and/or cryopreservation needs to be modified to restore viability of the avian hepatocytes.

CONCLUSION

Avian primary CEF and CK cells could be stored successfully in liquid nitrogen for a period up to four years. The cells when revived periodically and seeded into the cell culture flasks or plates formed confluent monolayer within two to three days. A method for cryopreservation of avian primary cells was standardized and tested.

ACKNOWLEDGEMENTS

The authors are highly grateful to Mr. James Wallis, President of Hygieia Biological Laboratories for providing funds and facilities to carry out this study.

REFERENCES

1. Buranathai C., Rodriguez J., Grose C. Transformation of Primary Chicken EmbryoFibroblasts by Marek's Disease Virus. Virology. 239:20-35; 1997.

2. Choi JW., Shin EK., Ha SH., Kim H.A., Kim Y.H., Kim S.S., Hahn TW. OptimalConditions for Cryopreservation of Primary Chicken Embryo Kidney Cells with Dimethyl Sulfoxide. In: Basile G.(editor-in-chief). Molecular Biotechnology. 35:237-241; 2007.

3. Chomiak T.W., Luginbuhl R.E., Helmboldt C.F. Tissue Culture and Chicken EmbryoTechniques for Infectious Laryngotracheitis Virus Studies. Avian Diseases. 4:235-246; 1960.

4. Cotter C.A., Earl P.L., Wyatt L.S., Moss B. Preparation of Cell Cultures and VaccinaVirus Stock. Current Protocols in Microbiology. 117: 16.16.1-16.16.18; 2017.

5. Fadly A.M. Isolation and Identification of Avian Leukosis Viruses: A Review. In: BlakeD. (editor in chief). Avian Pathology. Taylor and Francis. 29: 529-535; 2000.

6. Freire M.S., Mann G.F., Marchevsky R.S., Yamamura A.M.Y., Almeida L.F.C., Jabor A.V., Malachias J.M.N., Coutinho E.S.F., Galler R. [Production of Yellow Fever 17DD Vaccine Virus in Primary Culture of Chicken Embryo Fibroblasts: Yields, Thermo and Genetic Stability, Attenuation and Immunogenicity.Elsevier. 23:2501-2512. Portuguese. 2005.

7. Hennion R.M., Hill G. The Preparation of Chicken Kidney Cell Cultures for Virus Propigation. In: Maier H., Bickerton E., Britton P. (editors). Coronaviruses: Methods and Protocols, Methods in Molecular Biology. Humana Press New York. 1282:57-62; 2015.

8. Hernandez R., Brown D.T. Growth and Maintenance of Chick Embryo Fibroblasts (CEF). Current Protocols in Microbiology. 17: A.4I.1-A.4I.8; 2010.

9. Jackwood M.W., de Wit S. Infectious Bronchitis. In: David E. Swayne, editor. Diseases of Poultry. John Wiley & Sons. 13:139-143; 2013.

10. Jones R.C. Reovirus Infections. In: David E. Swayne, editor. Diseases of Poultry. John Wiley & Sons. 13:351-366; 2013.

11. Kohn F.S., Henneman S.A. Preparation of Primary Chicken Embryo Liver Cells. Tca Manual. 1:25-26; 1975.

12. McManus A.T., Parker D.R., Kenyon R.H., Kondig J.P., Eddy G.A. Use of Frozen Chicken and Duck Embryo Cells for Plaque Assays of Arboviruses and Rickettsiae. Journal of BiologicalStandardization. 2:329-332; 1974.

13. Myers T.J., Schat K.A. Propagation of Avian Rotavirus in Primary Chicken Kidney Cell and MA104 Cell Cultures. Avian Diseases. 33:578-581; 1989.

14. Sabin A.B., Arechiga A.F., Fernández de Castro J., Sever J.L., Madden D.L., Shekarchi I., Albrecht P. Successful Immunization of Children With and Without Maternal Antibody by Aerosolized MeaslesVaccine. JAMA, 249:2651-2662; 1983.

15. Ratnamohan N., Spradbrow P.B. Preservation of Avian Cells at Sub-zero Temperatures.Australian Journal of Biological Sciences. 32:475-481; 1979.

16. Salauddin M. A Brief Concept of Cell Culture: Challenges, Prospects and Applications [Online First, accessed January 18, 2022]. IntechOpen; 2021. doi:10.5772/intechopen.99387.

17. Yamaguchi T., Setiyono A., Kobayashi M., Takigami S., Kukushi H., Hirai K. InfectiousBursal Disease Live Vaccines: Changes in the Virus Population During Serial Passage in Chicken Embryo Fibroblast Cells. Avian Diseases. 44:284-290; 200.

RE-EMERGENCE OF CHONDRONECROSIS WITH OSTEOMYELITIS SYNDROME IN BROILERS AND THE EFFECT OF DIETARY PROBIOTICS IN A *STAPHYLOCOCCUS AGNETIS* CHALLENGE MODEL

J. Schleifer[A], A. Al-Rubaye[B], N.S. Ekesi[B], A. Hasan[B], D.A. Kolte[C], R. Wideman, Jr.[B], and D.D. Rhoads[B]

[A]Chr. Hansen, Inc., Milwaukee, WI
[B]University of Arkansas, Fayetteville, AR
[C]University of Baghdad, Baghdad, Iraq

SUMMARY

Bacterial chondronecrosis with osteomyelitis syndrome (BCOS) has re-emerged as an important cause of lameness in broilers in North America. This lameness is a significant animal welfare issue facing the North American broiler industry. Different bacteria have been associated with the pathogenesis of the disease. Those bacteria include *Enterococcus cecorum, E. coli*, *Staphylococcus agnetis* and others. It is generally accepted that the mode of entry of the bacteria is due to translocation from the gastro-intestinal tract or the respiratory system into the cardiovascular system. Other contributing factors to the pathogenesis are associated with rapid growth of the skeletal system and stress on the bird. Mitigating lameness due to BCOS is of intense interest and has been extensively investigated.

The continuous use of dietary probiotics has demonstrated an effect on reducing lameness in experimental challenge studies. BCOS investigations conducted at the University of Arkansas, using a well-documented challenge model, support the strategy of using dietary probiotics to mitigate BCOS-related lameness.

Data from this study will be presented showing a statistically significant ($P < 0.05$) reduction in lameness as a result of a *Staphylococcus agnetis* challenge model when compared to similarly challenged cohort broilers not fed a dietary probiotic. The reduction in lameness observed in this study is most pronounced after 43 days-of-age.

INTRODUCTION

The incidence of bacterial septicemic-associated lameness appears to be on the increase in the United States in the last 18-24 months. Numerous reports have been received of flocks showing symptoms consistent with Bacterial chondronecrosis with osteomyelitis (BCO) lameness.

The proposed pathogenesis of a bacterial origin resulting in lameness has been discussed for numerous years. However, the most recent and widely-supported pathogenesis has been reported that pathogenesis involves the occurrence of microfractures in rapidly growing bones of broilers (1, 2). This is coupled with stress and the exposure to a bacterial pathogen which translocates across the intestinal barrier or through the respiratory system. Different bacteria have been isolated in pure culture associated with the disease. Of note are the following bacteria: *Enterococcus cecorum, E. coli*, and *Staphylococcus* spp. (3) induced BCO-related lameness with a specific strain of *Staphylococcus agnetis* identified as 908. This strain was introduced to susceptible broilers through the drinking water.

Mitigation of BCO lameness has been reported. Associated with this paper is the report by Wideman (2) indicating mitigation of BCO-related lameness with a probiotic or a probiotic/prebiotic combination. We are reporting a summary of recently published paper (4). A mitigating effect on BCO with the dietary feeding of two different probiotics was observed in this study. Additionally, the *Staphylococcus agnetis* 908 challenge was introduced by way of drinking water in one treatment group with the suspected aerosol spread of the agent to "down-wind" treatment groups.

MATERIALS AND METHODS

The study was conducted at the University of Arkansas poultry research facilities. Four hundred fifty day-old surplus broiler breeder males were placed in 1.5 x 3 m pens on fresh litter. Housing and husbandry of the birds were according to reasonable and customary industry standards regarding feeding, ventilation, water delivery and temperatures. The birds were divided into four different treatments of 50 birds per pen. Treatment pens were allocated using a randomized block design. The treatments are identified in Table 1.

The administration of the probiotic in treatments 3 and 4 were included in all diets starting at one day-of-age and continued until the conclusion of the trial at 56d. The Source treatment group (Treatment 1) was challenged in the drinking water on days 20 and 21 with *Staphylococcus agnetis* 908. This strain has been previously identified as a bacterium which can induce BCO (3). The pens challenged with *S. agnetis* were located "upwind" from all the other pens so the ventilation flow would be from the Source to the treatment pens (control or probiotic).

Starting at 20d all birds were encouraged to move twice per day using a kitchen broom. Birds hesitant to move were marked with spray paint. Birds that were unable to walk were diagnosed as clinically lame and euthanized. Assessment of lameness was determined daily by a trained technician, blinded to the treatment allocations. Necropsies were performed on euthanized birds to record and assess bone lesion severity. For surviving birds, body weight gain was determined at 56d on a random selective weighing of eight birds.

RESULTS

Lameness was achieved with the *Staphylococcus agnetis* 908 strain challenge model as delivered through the drinking water with birds raised on new litter. The unchallenged control showed a resulting percentage of lame birds/pen of 64%. This compares to the challenged source treatment group demonstrating a 77% incidence of lameness (Table 1). The two treatments receiving the probiotic showed a reduction of lameness incidence when compared to the control (unchallenged) group. The final incidence of lameness for the DSM 17236 probiotic and the DSM 17299 probiotic treatments were 48% and 51% respectively. Lameness was reduced by 25% by DSM 17236 probiotic and 20% by DSM 17299 probiotic. No significant difference was noted in lameness reduction when comparing the 56d cumulative results between probiotic treatments.

The cumulative progression of lameness for all four treatments is illustrated in Figure 1. Visible lameness generally became apparent at 39d. At 43d the lameness incidence accelerated in the Source treatment compared to the other three treatments. The incidence of lameness remained noticeably elevated in the Source treatment throughout the entire trial. A dramatic increase in lameness incidence was observed in the Control (non-oral challenged) group at 55d. The cumulative progression of lameness remained relatively consistent in the two treatments fed the probiotics throughout the trial.

The heaviest average body weights were observed in the Source treatment (Table 2). The two treatments containing the probiotic showed the next heaviest average weights. A statistically significant difference in 56d body weights were observed between the Source treatment and the Control. A statistical difference was also observed between the Source treatment and the treatment fed the DSM 17299 probiotic.

DISCUSSION AND CONCLUSION

The induction of lameness with the *Staphylococcus agnetis* 908 challenge model demonstrated the onset of lameness at approximately 39d. This is consistent with previous reports either using this bacterial strain or using a stress model such as wired flooring, (1, 2, 3). The incidence of lameness in the Source birds is consistent with levels achieved in other challenge models reported in the manuscripts. This level of lameness indicates that the drinking water, "up-wind" challenge model with *Staphylococcus agnetis* 908 is a viable challenge model for the induction of BCO-related lameness.

The reduced incidence as well as the delay in the acceleration of lameness symptoms in the Control group also is supported by the references sited. The reduced incidence of lameness in the probiotic treated birds and the lack of lameness acceleration in the 43d period is a logical conclusion as these treatments were challenged similarly to the Control group. The absence of a direct *Staphylococcus agnetis* 908 challenge is illustrated with the reduced incidence of lameness in these treatments. Of interest is the spike in lameness in the Control group at 55d. This was not observed in either of the treatments fed a probiotic. The dietary inclusion of a probiotic or a probiotic/prebiotic combination has been reported to mitigate BCO-related lameness (2). In the report by Wideman (2) the probiotic-treated groups were directly challenged through a rigorous stress model. The reduced lameness at the end of the trial phase is of interest and additional study is needed to determine the significance.

The increased average body weight at 56d in the Source group is worth noting. The researchers suggest this increased weight could be associated with the extreme disease pressure on the Source birds. This pressure could select for more robust birds. Alternatively, the increased lameness level in this treatment group could result in fewer birds demanding feeder space; ultimately allowing the ambulatory birds increased feed access and consumption. Feed efficiency was not reported in this trial. The aspect that only 8 birds were weighed per treatment could have resulted in a statistical artifact. The increased body weights in the probiotic treatments is a logical conclusion based on widely-known influences some probiotics have on performance as well as the

observed lower incidence of lameness in the last few days of the trial.

The daily feed inclusion of the two probiotics used in this trial did result in a numerical mitigation of BCO-related lameness. This mitigation is consistent with previously published reports on the mitigating effect of other selected probiotics or probiotic/prebiotic combinations. The various modes-of-action of these select products appear to have a reproducible effect. The probable mode-of-action resulting in this effect could be; competitive exclusion, direct pathogen antagonism through bacteriocin production, improved intestinal barrier function, and enhanced immune function in the host animal.

REFERENCES

1. Wideman RF, Hamal KR, Stark JM, Blankenship J, Lester H, Mitchell KN, Lorenzoni G, Pevzner I. A wire-flooring model for inducing lameness in broilers: evaluation of probiotics as a prophylactic treatment. *Poult Sci.* 2012.

2. Wideman RF. Bacterial chondronecrosis with osteomyelitis and lameness in broilers: a review. *Poult Sci*. 2016.

3. Al-Rubaye AAK, Couger MB, Ojha S, Pummill JF, Koon JA II, Wideman RF Jr , Rhoads DD. Genome analysis of Staphylococcus agnetis, an agent of lameness in broiler chickens. *PLoS One 10:e0143336*. 2015.

4. Al-Rubaye AAK, Ekesi NS, Hasan A, Koltes DA, JF, Koon, II JA, Wideman RF Jr, Rhoads DD. Chondronecroisis with osteomyelitis in broilers: further defining a bacterial challenge model using standard litter flooring and protection with probiotics. *Poult Sci.* 2020.

Table 1. Treatment designations and results of total lame and percent lame as identified for each individual pen and averaged for the treatments through 56d.

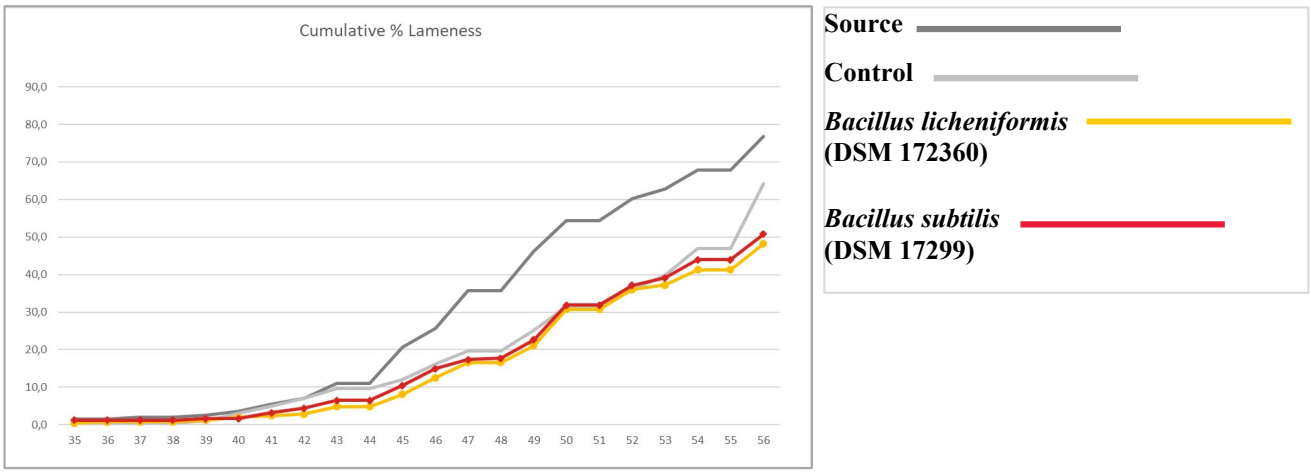
Treatment number	Treatment	Description	Average lame count/pen	Percentage of birds demonstrating lameness/pen
1	Source	Control diet with Staphylococcus agnetis 908 10^5 CFU/ml in drinking water on days 20 & 21	38.3 ± 2.5	77
2	Control	Control diet	31.8 ± 2.4	64
3	*Bacillus licheniformis* probiotic (DSM 172360)	DSM 17236 probiotic 3.2 x 10^9 CFU /g of product at 0.5 kg/metric ton of feed	23.8 ± 1.1	48
4	*Bacillus subtilis* probiotic (DSM 17299)	DSM 17299 probiotic 1.6 x 10^9 CFU /g of product at 0.5 kg/metric ton of feed	25.2 ± 2.5	51

Table 2. Average body weights of 8 birds per treatment at 56d.

Treatment	Average BW kg ± SEM	*P*-value[1] Control	*B. licheniformis*	*B. subtilis*
Source	4.40 ± 0.05	0.021	0.17	0.047
Control	4.14 ± 0.07		0.17	0.34
***Bacillus licheniformis* probiotic (DSM 172360)**	4.27 ± 0.10			0.28
***Bacillus subtilis* probiotic (DSM 17299)**	4.19 ± 0.10			

[1] *P*-value computed by a T-test comparing the treatment in the first column to each of the other treatments.

Figure 1. Comparison of cumulative lameness of birds raised on litter when challenged with *Staphylococcus agnetis* 908, or unchallenged as described in Table 1.



ROLE OF TURKEY HEPATITIS REOVIRUSES IN TENOSYNOVITIS IN TURKEYS

T. Sharafeldin[A], B. Hause[A], and R. Rink[B]

[A]Department of Veterinary and Biomedical Sciences, Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, SD 57007
[B]Dakota Provisions, Huron, SD 57350

INTRODUCTION

Since the beginning of 2019, several cases of hepatitis associated morbidity and mortality (ranging between 1%-5%) were seen in turkey poults 10-25-day-old. After ruling out all possible pathogens, only reovirus was confirmed to be associated with this problem. A previous experimental trial reproduced the hepatic lesions and concluded that turkey hepatitis reoviruses are likely to induce tenosynovitis at an older age.

PROCEDURE

A flock of 18-day-old turkey poults had 5% lethargy and 1% mortality in a few days. Dead birds had multifocal necrotic hepatitis and reovirus isolation was confirmed. This flock was observed up to the marketing age.

RESULTS

At six weeks of age, a sample of leg tendons from that flock was examined histologically, and a remarkable tenosynovitis was observed and reovirus was observed. This flock grew in a farm with a history of turkey arthritis reovirus in other flocks in other houses in the same farm. The other flocks had no hepatitis at a young age but had tenosynovitis only and they showed finishing parameters better than this flock that encountered hepatitis at a young age. A metagenomic analysis is conducted to compare these turkey reovirus isolates from different flocks. Initial complete genome revealed that the reovirus isolated from livers and from tendons of that flock were nearly identical. Further analysis results are to be presented.

CONCLUSION

This is the first field investigation to show the likelihood of turkey hepatitis reovirus to induce tenosynovitis at an older age in the same flock.

EXPERIENCES WITH INCLUSION BODY HEPATITIS IN THE SOUTHEAST UNITED STATES

E. Shepherd, D. French, J. Nicholds, K. Grogan, H. Sellers, and S. Williams

PDRC, University of Georgia, 953 College Station Road, Athens, GA 30602

SUMMARY

Inclusion body hepatitis (IBH) has been a consistent issue in the southeast United States (US) for the last few years. There has been a recent shift towards serotype 8b being predominantly isolated in affected broiler flocks but serotypes 5, 8a, and 11 have also been found. The majority of flocks were typically affected between two to four weeks of age and have had daily mortality range from minor (0.1%) to very significant (1-2%). Affected broiler integrators have been able to control IBH using various interventions including, but not limited to, autogenous vaccine development, cleaning and disinfection of houses, litter removal and heat treatment, and control of immunosuppressive diseases. Anecdotal information seems to indicate that the companies using autogenous vaccines in their broiler breeders have had a greater amount of success controlling IBH than those companies who did not use autogenous vaccines. While there is still debate if IBH is a result of a primary or secondary pathogen, our integrator questionnaire results and University of Georgia Poultry Diagnostic Research Center (UGA PDRC) submissions suggest that serotype 8b may be a primary pathogen with nearly 83% of submissions being serotype 8b.

INTRODUCTION

Inclusion body hepatitis (IBH) is caused by a double stranded DNA virus that belongs to the genus Aviadenovirus of the Adenoviridae family (formerly identified as group 1). There are several viruses included in this family, some considered primary pathogens while others considered secondary. Other notable poultry pathogens in this genus include quail bronchitis and hydropericardium syndrome. Siadenovirus (formerly Group 2) is responsible for marbled spleen disease and hemorrhagic enteritis while Atadenovirus (formerly Group 3) causes egg drop syndrome (1). The viruses are hardy and resistant to lipid solvents and changes in pH between 3 and 9. They are also able to survive temperatures up to 70°C (158°F) for up to 30 minutes (2). This resistance to elevated temperatures makes degradation in commercial poultry houses virtually impossible, even with windrowing. These viruses form basophilic intranuclear inclusion bodies making diagnosis by histopathology routine (Figure 1). Gross lesions in livers are said to be of a "ground glass" or "sandy beach" appearance, and while not pathognomonic itself, are highly suspicious of the disease when coupled with age of onset and mortality pattern (Figure 2). Polymerase chain reaction (PCR) targeting the hexon gene and virus isolation in cell culture are also used (3). Positive samples are further sequenced and those isolates may be saved for autogenous vaccine usage.

Fowl adenoviruses (FAV) are classified into five species (A-E) based on whole genome sequences. Strains belonging to FAV group D and E can cause severe liver damage resulting in what we clinically call IBH (2). Mortality associated with IBH tends to occur for about five days following clinical presentation and can be quite sudden (2). Mortality can range from minor to catastrophic with some cases in Australia reaching 30% (4). Incubation period is short at 24 to 48 hrs with infection via natural routes and spreads quickly inside an affected house via the fecal oral route. There is no specific treatment for IBH, but activated vitamin D and electrolytes in the water are typically used to minimize bone issues, such as rickets, later in the flock.

Since adenoviruses are quite hardy, they can stick around in houses for a long time and create "repeater houses" where subsequent flocks also experience mortality associated with IBH. This can be frustrating for integrators as they try to combat this problem with limited tools at their disposal.

The aim of this study was to learn to what extent IBH was an issue for integrators in the southeast US and the control measures that worked best for them.

MATERIALS AND METHODS

In order to ascertain the impact of IBH on poultry companies and how it is being best controlled, a verbal questionnaire was used in which five companies in the southeast US responded. The following six questions were asked:

1) Have you had any IBH cases in the last year?
2) If so, what was the clinical presentation? Did you have repeater houses?

3) What serotypes did you isolate?
4) Did you control IBH in your complex using autogenous vaccine?
5) Were there any other control measures used besides vaccine?
6) Is IBH currently an issue for you?

Data from submissions to UGA PDRC from 1-5-2021 to 1-4-2022 were compiled and positive cases (either by virus isolation and/or PCR with sequencing) were reported. Negative samples or positive samples where sequencing was not requested were not reported. These submissions included both domestic and international samples.

RESULTS

Results from the integrator questionnaire are summarized below in Table 1 and UGA PDRC submissions were tabulated and listed below in Table 2. All five companies that participated had at least one case of IBH in the last year. All five of the companies isolated serotype 8b while only two isolated serotype 11. This mirrors the UGA PDRC submission data with nearly 83% of positive cases being serotype 8b while serotype 11 was isolated just 12% of the time. Age at onset of clinical IBH was divided into two distinct age groups. The first group was about 2.5 weeks old (16 days) and the other group was about four weeks old (26 days). Three companies saw mortality spikes at both time points while the other two companies only saw mortality in the older group. Four companies believed that their IBH cases were primary (the result of vertically shedding breeder flocks) while one company believed it was due to immunosuppressive diseases (primarily infectious bursal disease). Remediation of IBH was split between the companies with three companies developing an autogenous vaccine and two companies relying on other intervention strategies.

DISCUSSION

The data from the UGA PDRC submissions and our questionnaire show that the predominant serotype detected in clinical IBH cases in the southeast US was largely FAV E/ FAdV 8b and FAV D/FAdV 11. These findings are similar to what was found in a recent 10-year retrospective study of broiler IBH cases in Spain where FAdV 8b was found 63.6% of the time and FAdV 11 was found 32.4% (5). The worldwide distribution of these serotypes, especially 8b, provides further evidence that this serotype is most likely a primary pathogen of chickens resulting in significant morbidity and mortality in affected flocks. It is important for integrators to maintain good surveillance for IBH in their operations and continue to gather isolates for possible autogenous vaccine usage to help minimize the impact of this disease in their operations. Autogenous vaccines take time to be manufactured and then time must pass until maternal antibodies are developed and reach the progeny being placed in the field.

This study showed that other mitigation strategies, including removal of used litter, windrowing litter, and cleaning and disinfection of these houses can also be used effectively.

REFERENCES

1. Fitzgerald S D. Adenovirus Infections. In: Diseases of Poultry, 14th edition, Swayne D E, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL., eds. Wiley-Blackwell, USA, pp 321-322. 2020.

2. Hess M. Aviadenovirus Infections In: Diseases of Poultry, 14th edition, Swayne D E, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL., eds. Wiley-Blackwell, USA, pp 322-332. 2020.

3. Benko M, Harrach B, and Russell WC. Family Adenoviridae. In Van Regenmortel M H V, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (eds.). Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press: New York and San Diego. pp 227–238. 2000.

4. Barr D A and Scott P. Adenoviruses and IBH. Proc 2nd Asian/Pacific Poult Health Conference [Proc 112]. Post Graduate Comm Vet Sci, University of Sydney, Australia, pp 323–326. 1988.

5. Bertran K, Blanco A, Antilles N, Nofrarías M, Valle RM, Cobos À, Ramis A, Biarnés M, Majó N. A 10-Year Retrospective Study of Inclusion Body Hepatitis in Meat-Type Chickens in Spain (2011–2021). Viruses. 13, 2170. https://doi.org/10.3390/v13112170. 2021.

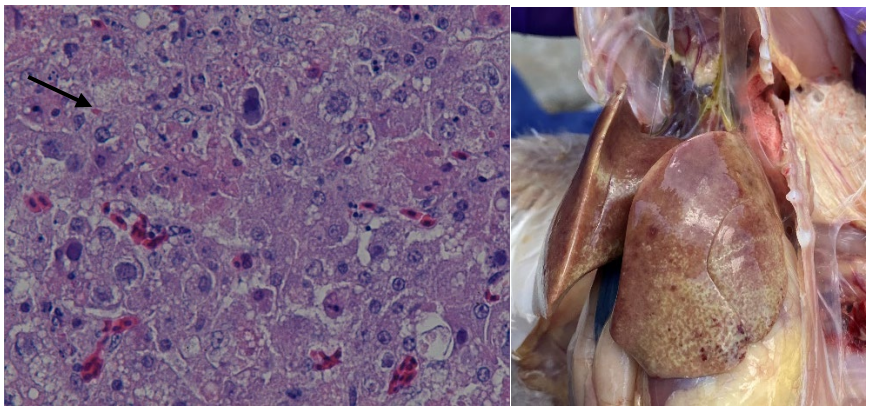
Table 1. Integrator questionnaire.

Question	Company A	Company B	Company C	Company D	Company E
IBH in the last year?	Yes	Yes	Yes	Yes	Yes
Age at onset? Repeater houses?	2.5wks and 4wks. Repeater farms.	2.5wks and 4wks. Repeater farms.	4wks. No repeaters.	2.5wks and 4wks. Repeater farms.	4wks. No repeaters.
Serotype(s) isolated?	8b only	8b and 11	8b and 11	8b only	8b only
Autogenous vaccine used?	Yes	Yes	Yes	No	No
Other control measures?	Clean out and C&D	Clean out, C&D, and heating houses	No	Clean out, windrow, and C&D	IBDV control
IBH still an issue?	No	Yes	No	No	No

Table 2. UGA PDRC submission results.

Total Positive Submissions		FAdV 5 (% positive)	FAdV 8a (% positive)	FAdV 8b (% positive)	FAdV 11 (% positive)
357		3 (0.8%)	15 (4.2 %)	**295 (82.6%)**	44 (12.3%)

Figure 1 (left). Intranuclear inclusion bodies (denoted by arrow) (40x mag).
Figure 2 (right). IBH gross lesion in liver.



A SYSTEMATIC REVIEW OF INTERVENTIONS FOR PREVENTING AND TREATING NECROTIC ENTERITIS IN BROILER CHICKENS

R. Singer[A], I. Bueno[A], H. Hwang[A], I. Ricke[B], E. Smith[A], G. Moores[A], A. Nault[C], and R. Valeris-Chacin[A]

[A]Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota
[B]Mindwalk Consulting Group, LLC
[C]Veterinary Medical Library, College of Veterinary Medicine, University of Minnesota

SUMMARY

Necrotic enteritis (NE) is one of the most common and economically important diseases affecting the broiler industry. Limitations on the use of antimicrobials have highlighted the need to evaluate the efficacy of non-antibiotic alternatives and management strategies. We therefore conducted a systematic review to address the question: *What is the efficacy of non-antibiotic interventions, compared to antibiotic interventions, in preventing and treating necrotic enteritis in broiler chickens?* A comprehensive search of the databases PubMed/MEDLINE, CAB Abstracts, Agricola, and Scopus was conducted returning 1,282 citations. Titles and abstracts were screened for relevance, resulting in 536 included studies. The methods of these studies were then screened, resulting in a total of 171 studies from which data were extracted. These studies are being evaluated for biases. Results will indicate the current evidence to answer the study question and will highlight data gaps that need to be addressed.

INTRODUCTION

Necrotic enteritis (NE) is one of the two most important diseases of chickens requiring antimicrobial therapy in the United States (U.S.) (1). However, rising concerns about antimicrobial resistance combined with changes to antimicrobial use practices within the poultry industry have resulted in investigations of alternatives to antibiotics to prevent and treat NE. A comprehensive review of available evidence about the efficacy of such alternatives has not been conducted to date. Therefore, the goal of this study was to conduct a systematic review of the literature to critically evaluate the available evidence on the following research question: *What is the efficacy of non-antibiotic interventions, compared to antibiotic interventions, in preventing and treating necrotic enteritis in broiler chickens?*

METHODS

A protocol for this systematic review was created *a priori* and registered in the University of Minnesota Digital Conservancy (2). The protocol was also published online at the Systematic Reviews for Animals and Food (www.syreaf.org). This review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (3). Briefly, a search strategy involving the databases PubMed/MEDLINE, CAB Abstracts, Agricola, and Scopus was conducted. All citations found through this search were screened in two steps. First, titles and abstracts were evaluated, excluding studies that were not relevant to the review question. Second, the full-text of included studies from the first screening step was retrieved and the methods section evaluated. Data consisting of general study characteristics and study details such as intervention type or necrotic enteritis detection methods and outcome data were then extracted from the studies that had been selected through the screening process. Individual outcomes for each included study were also assessed for risk of bias based on a modified protocol (4), classifying these outcomes into 'high', 'low', or 'some concerns' for the risk of bias assessment. For each of the review steps, two independent reviewers evaluated each study and reached a decision about each paper through weekly meetings. Data from the review are being analyzed and summarized to assess the efficacy of antibiotic interventions compared to non-antibiotic interventions.

RESULTS

A total of 1,282 records were retrieved from all databases and evaluated for relevance screening. After the two-step screening and data extraction 171 papers were selected into the review (Fig. 1). Studies in the review were from 63 different journals, being the most common ones *Poultry Science* (26%), *Avian Pathology* (8%), and *Avian Diseases* (7%), and most studies were published between 2010 and 2019 (58%).

Studies were conducted in a total of 23 countries, most frequently in the U.S., Australia, and Canada. Most included studies used an experimental study design, with only two studies being observational. Broiler breed was reported in 93% of the studies, and the most common breeds were Ross (44%), Cobb (22%), and Arbor Acres (7%). Forty-one studies compared at least two different interventions (e.g., antibiotic and prebiotic) while 130 studies evaluated only one type. The antibiotics most commonly evaluated across all studies were amoxicillin, avilamycin, avoparcin, bacitracin, lincomycin, penicillin G, salinomycin, tylosin, and virginiamycin. The non-antibiotic alternatives included prebiotics, probiotics, vaccines, and other (e.g., oils, anticoccidial drugs, organic acid mixtures, and polymers). The most commonly reported outcome to measure NE were lesion scores (n=100 studies), and 134 studies reported more than one type of measured outcome (e.g., NE lesion scores and *Clostridium perfringens* counts). A preliminary assessment from this review shows no compelling evidence for a higher efficacy of non-antibiotic alternatives compared to antibiotics used to prevent or treat NE. The risk of bias assessment evaluated a total of 388 outcomes for the 171 studies. Of these, 36% of outcomes were classified as 'high', 64% as 'some concerns', and none of the outcomes were classified under the 'low' category.

DISCUSSION

This systematic review summarized the available evidence assessing the efficacy of non-antibiotic alternatives in the management of NE in broiler chickens. Studies evaluated were heterogenous in their intervention types as well as in the outcomes analyzed, which made them challenging to compare. In addition, some studies did not report any statistical analyses and/or epidemiological effect measures to inform the strength of the association between interventions and NE outcomes. Despite the current knowledge gaps and potential improvements in design and reporting for future studies, there is a relevant and increasing body of knowledge assessing non-antibiotic alternatives to prevent and treat NE, which will be critical to manage this important disease for the poultry industry.

(A full-length article will be published in a peer-reviewed journal in the near future.)

ACKNOWLEDGEMENTS

Funding for this project was made possible, in part, by grant no. 2015-68003-22972 from the USDA National Institute of Food and Agriculture and grant no. U01FD005878 from the U.S. Food and Drug Administration.

REFERENCES

1. Singer RS, Porter LJ, Schrag NF, Davies PR, Apley MD, Bjork K. Estimates of on-farm antimicrobial usage in broiler chicken production in the United States, 2013–2017. Zoonoses Public Health, 67:22-35; 2020. https://doi.org/10.1111/zph.12764.

2. Bueno I, Smith E, Hwang H, Nault AJ, Valeris-Chacin R, Singer RS. Efficacy of antibiotic and non-antibiotic interventions in preventing and treating necrotic enteritis in broiler chickens: a protocol for a systematic review. 2020. https://conservancy.umn.edu/handle/11299/216460.

3. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, Shamseer L, Tetzlaff JM, Akl EA, Brennan SE, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ. 372; 2021. https://doi.org/10.1136/bmj.n71.

4. Winder CB, Sargeant J M, Hu D, Wang C, Kelton DF, Leblanc SJ, Duffield F, Glanville J, Wood H, Churchill KJ, Dunn J, et al. Comparative efficacy of teat sealants given prepartum for prevention of intramammary infections and clinical mastitis: a systematic review and network meta-analysis. Anim. Health Res. Rev. 20:182-198; 2019. https://doi: 10.1017/S1466252319000276.

CPG-ODN-INDUCED ANTIMICROBIAL IMMUNITY INVOLVES IMMUNO-METABOLIC REGULATION IN BROILER CHICKENS

I. Subhasinghe[A], K. Ashfaque Ahmed[A], H. Gautam[A], A. Shayeganmehr[A], S. Popowich[A], B. Chow-Lockerbie[A], and S. Gomis [A*]

[A] Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada

*Corresponding author: Email: susantha.gomis@usask.ca

ABSTRACT

Synthetic short segments of DNA (oligodeoxynucleotides containing Cytosine phosphodiester Guanine motifs (CpG-ODNs) represent a type of pathogen-associated molecular pattern (PAMP) that can stimulate immune cells and promote antimicrobial immunity. Activated immune cells require profound metabolic changes to meet cellular signaling process and biosynthesis demands. We hypothesize that the stimulation of immune cells by CpG-ODNs promotes profound metabolic programming to facilitate the antimicrobial activities of immune cells. We administered CpG-ODN or saline into two-week-old broiler chickens via intramuscular route and longitudinally evaluated immunometabolic responses in the blood leukocytes. In this study, we used flow cytometry-based methods to assess the antimicrobial activities of chicken heterophils. The metabolic status of immune cells was evaluated in real-time using the Seahorse metabolic analyzer. We observed higher ($p<0.001$) antimicrobial activity in heterophils, and enhanced cellular metabolic capacity (mitochondrial respiration and glycolysis) in CpG-ODN injected groups than saline controls. Our study provides the first evidence for the immunometabolic regulation of CpG-ODN-induced antimicrobial immunity in broiler chickens.

INTRODUCTION

The commercial poultry industry seeks effective strategies against bacterial and viral challenges that can cause increased mortality and morbidity and decreased production. As growth-promoting agents, prophylactic antibiotics in-feed have been used for a long-time to prevent bacterial infection in poultry production. Usage of antimicrobials, especially antibiotics in animal production, led to the emergence of antibiotic-resistant strains of bacterial pathogens (1). Antimicrobial resistance (AMR) is a quintessential global one-health concern. Therefore, the poultry industry is urgently looking for alternatives to antibiotics (2, 3). Oligodeoxynucleotides containing cytosine-phosphodiester guanine motifs (CpG-ODN) is a proven immune stimulant in chickens, which is recognized by toll-like receptor (TLR)-21 in the avian immune system (4). Our previous studies provided strong evidence that CpG-ODN delivery in chickens via several routes, such as intramuscular (IM), subcutaneous (SC), in-ovo, intraperitoneal (IP), and intrapulmonary (IPL), can protect broiler chickens against lethal bacterial infections, including *E.coli* and *Salmonella* (4-8).

Major antimicrobial activities of innate immune cells, including heterophils, can be recognized by investigating phagocytosis, oxidative burst and degranulation. Chicken heterophils are functional homologous to mammalian neutrophils and an integral part of the first line of host defense in chickens (9, 10). These antimicrobial functions are stimulated in response to threat/danger signals from infectious pathogens. However, immune activation is an energy-demanding process to increase cytokine production, nucleotide synthesis, and cellular biosynthesis. Therefore, immune cells must undergo a profound metabolic change by altering their aerobic glycolysis and mitochondrial respiration pathways. Quantification of these pathways and mechanisms provide significant insights into the CpG-ODN mediated immune protection, which may help utilize the full potential of CpG-ODN as an alternative to antibiotics in chickens. In this study, we focused our investigation on the antimicrobial activities of chickens heterophils and immunometabolic regulation of chicken immune cells in chickens. To the best of our knowledge, this study provides, for the first time, evidence for the CpG-ODN-induced immunometabolic regulations at the cellular level.

MATERIALS AND METHODS

Stimulation of leukocytes in chickens by administering CpG-ODN. The CpG-ODNs ($TCGTCGTTGTCGTTTTGTCGTT_{2007}$), free of endotoxin and produced with a phosphorothioate

backbone were synthesized commercially (Operon Biotechnologies Inc., Huntsville, AL). Synthetic CpG-ODN was diluted in sterile, nonpyrogenic saline. CpG-ODN (50µg/bird) and saline (control group) were injected intramuscularly (IM) to 1-2 weeks old chicks. For the first experiment (antimicrobial functions of heterophils) blood samples were collected after 48 hours of CpG-ODN injections. For the second experiment, we had three groups of birds: G1- two CpG-ODN injections (three days apart), G2- one CpG-ODN dose, and G3- Control group. We collected blood samples at five different time points (24hrs, 48hrs, 7days, 14 days and 21 days) after CpG injections.

Quantification of antimicrobial activities of heterophils. Collected heparinized whole blood samples were processed in the laboratory (ex-vivo) using optimized functional assays. The oxidative burst activity of whole blood was measured using the Phagoburst® kit(Glycotope Biotechnology GmbH, Heidelberg, Germany), which was modified during optimization (11). Opsonized *Escherichia coli* and protein kinase-C ligand Phorbol 12-myristate 13-acetate (PMA) were used in this commercial kit. Leukocytes were stimulated with PMA for different periods (2, 5 and 10 minutes and optimized 5min as the greatest for heterophils burst activity) prior to adding dihydrorhodamine (DHR) 123 (fluorogenic substrate), which reacts with reactive oxygen species (ROS). Mouse anti-chicken CD45 (APC) fluorescent-labeled antibodies to detect all leukocytes and FCC and SCC were used to separate the heterophils population. A modified method of Phagotest® (Glycotope Biotechnology GmbH, Heidelberg, Germany) was used to examine phagocytosis. Briefly, leukocytes were stimulated with fluorescent (FITC) labeled opsonized *E. coli* for 10 minutes. Afterward, anti-chicken CD45 (APC) antibodies were added (12, 13). Degranulation of heterophils granulocytes was quantified using rabbit polyclonal to LAMP1 antibody - Lysosome Marker. Epics XL (Beckman Coulter) and FACS Caliber (BD Bioscience) were used to obtain flow cytometry data, and data were analyzed with FlowJo software (Tree Star).

Quantification of cellular glycolysis and mitochondrial respiration. Peripheral blood samples were collected and processed to isolate the peripheral blood mononuclear cell layer (PBMC). Cells were processed to measure aerobic glycolysis and oxidative phosphorylation (OXPHOS) using a Seahorse XFp (Seahorse Bioscience), and the cellular metabolism was measured in real-time.

RESULTS AND DISCUSSION

Birds who received CpG-ODN increased their oxidative burst activity by 50-60% compared to the control group who received no CpG-ODN (30-35%) ($P < 0.05$). Furthermore, the phagocytosis of CpG-ODN delivered birds (35-40%) was higher than the control birds (20-25%). An elevated level of degranulation was detected in birds who received CpG-ODN (35-38%) compared to the control group (12-14%). These experiments were repeated, and we have observed consistent results and repeatability of functions of heterophils. This study developed methods to evaluate chickens heterophils to assess oxidative burst, phagocytosis, and degranulation functions using flow cytometry. This study showed that CpG-ODN could enhance major antimicrobial activities in chicken heterophils.

Groups that received CpG-ODN had increased ($p<0.001$) mitochondrial respiration [oxygen consumption rate (OCR)] in G1 (two CpG-ODN doses) and G2 (One CpG-ODN dose) compared to G3 (Saline). Further, CpG-ODN injected group showed higher statistically significant mitochondrial respiration (OCR) than the saline group at 24 hours, 48 hours (the highest), day 7, and day 14-time points. Birds that received CpG-ODN had increased glycolytic capacity [extracellular acidification rate (ECAR)] in G1 and G2 compared to the control group. However, these results were statistically significant only at 48 hours time-point after CpG-ODN injections. The data indicate a substantial elevation of immune cell metabolism, especially in mitochondrial respiration. Our study demonstrated that CpG-ODN associated antimicrobial activity increased energy demand and metabolic activities of immune cells in broiler chickens. These findings will help improve the antimicrobial functions of CpG-ODN and develop a potent alternative to antibiotics for use in the poultry industry.

REFERENCES

1. Gyles CL. Antimicrobial resistance in selected bacteria from poultry. *Animal Health Research Reviews*. 2008;9(2):149.

2. Cervantes HM. Antibiotic-free poultry production: is it sustainable? *Journal of Applied Poultry Research.* 2015;24(1):91-7.

3. Diarra MS, Malouin F. Antibiotics in Canadian poultry productions and anticipated alternatives. *Frontiers in microbiology*. 2014;5:282.

4. Goonewardene KB, Popowich S, Gunawardana T, Gupta A, Kurukulasuriya S, Karunarathna R, et al. Intrapulmonary Delivery of CpG-ODN Microdroplets Provides Protection Against

Escherichia coli Septicemia in Neonatal Broiler Chickens. *Avian diseases*. 2017;61(4):503-11.

5. Gomis S, Babiuk L, Allan B, Willson P, Waters E, Ambrose N, et al. Protection of neonatal chicks against a lethal challenge of Escherichia coli using DNA containing cytosine-phosphodiester-guanine motifs. *Avian diseases*. 2004;48(4):813-22.

6. Gomis S, Babiuk L, Godson DL, Allan B, Thrush T, Townsend H, et al. Protection of chickens against Escherichia coli infections by DNA containing CpG motifs. *Infection and immunity*. 2003;71(2):857-63.

7. Gunawardana T, Foldvari M, Zachar T, Popowich S, Chow-Lockerbie B, Ivanova MV, et al. Protection of neonatal broiler chickens following in ovo delivery of oligodeoxynucleotides containing CpG motifs (CpG-ODN) formulated with carbon nanotubes or liposomes. *Avian diseases*. 2015;59(1):31-7.

8. He H, Lowry VK, Swaggerty CL, Ferro PJ, Kogut MH. In vitro activation of chicken leukocytes and *in vivo* protection against *Salmonella enteritidis* organ invasion and peritoneal S. enteritidis infection-induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide. *FEMS Immunology & Medical Microbiology*. 2005;43(1):81-9.

9. Genovese KJ, He H, Swaggerty CL, Kogut MH. The avian heterophil. *Developmental & Comparative Immunology.* 2013;41(3):334-40.

10. Kogut MH, Genovese KJ, Lowry VKJI. Differential activation of signal transduction pathways mediating phagocytosis, oxidative burst, and degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. *Inflammation*. 2001;25(1):7-15.

11. Parment K, Zetterberg A, Ernerudh J, Bakteman K, Steinwall I, Sjoberg F. Long-term immunosuppression in burned patients assessed by in vitro neutrophil oxidative burst (Phagoburst®). *Burns*. 2007;33(7):865-71.

12. Sawosz E, Winnicka A, Chwalibog A, Niemiec T, Grodzik M, Sikorska J. Phagocytic and oxidative-burst activity of blood leukocytes in rats fed a protein-free diet. *Bull Vet Inst Pulawy*. 2009;53:775-8.

13. Lun A, Schmitt M, Renz H. Phagocytosis and oxidative burst: reference values for flow cytometric assays independent of age. *Clin Chem*. 2000;46(11):1836-9.

A CASE OF NECROTIC ENTERITIS IN YOUNG BROILERS

R. Thiemann[A], J. Thornton[A], P. Stayer[B], E. Riley[B], R. Clark[B], N. Armour[A], and M. Pulido-Landínez[A]

[A]Poultry Research and Diagnostic Laboratory, Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University
3137 MS-468, Pearl, MS 39208
[B]Sanderson Farms, Inc.
P.O. Box 988, Laurel, MS 39411

ABSTRACT

Necrotic enteritis is an important intestinal disease of commercial poultry associated with poor performance, high mortality, and significant economic loss, particularly in the advent of NAE production. In this case report, necrotic enteritis is described in very young broilers. Clinically affected birds were submitted to the Poultry Research and Diagnostic Laboratory at Mississippi State University for further evaluation. Birds were between 5 and 11 days of age. Gross lesions reported by company veterinarians and observed during diagnostic necropsy included a moderate to severe enteritis of the upper small intestine, characterized by multifocal, irregular, plaque-like, mucosal ulcerations. More traditional lesions of necrotic enteritis, like thinning of the intestinal wall with consequential distension and ballooning and a necrotic, pseudomembranous layer covering the mucosa, were observed in some birds. Anaerobic culture was performed, and *Clostridium perfringens* was isolated from affected intestinal sections. All affected chicks originated from the same hatchery; despite being placed between two complexes. As such, environmental sampling at the hatchery was also performed to evaluate the presence and load of clostridial organisms. *C. perfringens* was isolated from samples collected in the egg room, hatchers/hatch halls, separator room, processing room, and transport trucks. Furthermore, VITEK® MS MALDI-TOF results indicated a 99.9% genetic relatedness between hatchery and live bird isolates, demonstrating an epidemiological link between clinically affected birds and the hatchery as a point source. The full-length article will be published in *Avian Diseases.*

INTRODUCTION

Commercial poultry are susceptible to several *Clostridium* species, including *C. perfringens, C. septicum, C. colinum,* and *C. botulinum.* The class *Clostridia* includes Gram-positive, spore-forming, rod-shaped, anaerobic bacteria belonging to the Family *Clostridiaceae* (1, 2). Its ability to form spores facilitates long-term survival and transmission in an aerobic environment (3). In the United States, the use of built-up litter, together with the shift away from the use of antibiotics and ionophores to achieve "no antibiotic ever" status has increased the prevalence and load of *Clostridium* in the commercial setting (4, 5, 6, 7). While exposure to *Clostridium* typically occurs in the bird's house environment, other critical points for exposure include the hatchery and feed from a bird health standpoint, as well as the processing plant from a food safety standpoint (8, 9, 10). Furthermore, fecal contamination of hatching eggs with *Clostridium* species has also been suggested as a route of transmission. Epidemiologic studies have shown persistence of identical ribotypes from breeder to broiler facilities within the same complex (11, 12).

Necrotic enteritis is an acute *C. perfringens* infection producing necrosis of the mucosal epithelium of the gastrointestinal tract, which often results in elevated mortality. Grossly, the small intestines are distended and friable, with necrosis of the mucosal surface of the intestines, forming a pseudomembrane (13). In 2000, necrotic enteritis was estimated to cost the global poultry industry two billion dollars annually due to its negative impact on livability, growth, and feed efficiency, as well as treatment and control costs (7).

Beginning in March of 2020, a Mississippi broiler integrator company experienced elevated mortality (7/1,000 chicks) during the first half of the production cycle on several broiler farms. Affected farms belonged to two complexes (complexes 1 and 2), separated by a distance of 25 miles. In total, 56 farms were affected from complex 1 and 10 farms from complex 2 over a 14-month period. Company veterinarians performed necropsies on fresh dead and found a high percentage of chickens presented with moderate to severe enteritis. White to gray, focal, plaque-like, 1 to 2 cm diameter ulcerations were present on the mucosal surface of the upper to middle gastrointestinal tract. In addition, some had gross lesions consistent with clinical necrotic enteritis. Externally, sectional ballooning and distention of

bowels with thinning of the intestinal wall was noted. Internally, the intestinal mucosa had a layer of tan to red-brown, diphtheritic debris covering its surface; a foul-smelling liquid content was present in the lumen in some cases. When the pseudomembranous layer was scraped away, gross ulcers were often revealed. Age of affected birds ranged from 5 to 45 days old. However, most cases occurred in young birds between 5 to 19 days of age. Three cases with similar presentations were submitted to the Poultry Research and Diagnostic Laboratory (PRDL) in Pearl, Mississippi. Affected birds were between 5 to 11 days of age. Flocks were reported to have uniformity issues, some bacterial infection, and weak and dehydrated birds. Ulcerative lesions were identified in the upper gastrointestinal tract from all submitted cases.

Despite affected farms being in two different complexes, all had sourced chicks from the same hatchery, located in complex 1. Between both complexes, coccidiosis control programs were identical and prophylactic antibiotics (Bacitracin Methylene Disalicylate; BMD) were included in the feed at the same dose and duration. Feed was formulated to be similar among all complexes, with any variation resulting from sourcing of feed ingredients. Based on this information and the early presentation of gross lesions and mortality in young birds, it was hypothesized that birds were exposed to the causative pathogen in the hatchery. Furthermore, preliminary histopathology findings of gram-positive, bacterial rods from sections of affected intestinal tract suggested the causative agent could be a *Clostridium* organism. Environmental sampling of the hatchery was performed in May of 2021 to isolate and culture this bacterium from the hypothesized common point source.

MATERIALS AND METHODS

Postmortem examination. Chicks were evaluated externally for signs of dehydration and improper navel healing. Birds were opened and evaluated for the presence of bacterial infection. Samples for bacteriology were aseptically collected using sterile, cotton-tipped swabs and streaked onto MacConkey and Blood (Tryptic Soy Agar (TSA) with sheep blood), Remel™ Thermo Fisher Scientific™, Lenexa, KS), and inoculated into Tetrathionate broth base (Remel™ Thermo Fisher Scientific™, Lenexa, KS), for *Salmonella spp* isolation following NPIP procedures. Additionally, anaerobic culture was performed in two of three cases by direct inoculation of 1-2 cm. lengths of ulcerated intestines on Thioglycolate media without dextrose (Remel™ Th ermo Fisher Scientific™, Lenexa, KS). Sections of affected gastrointestinal tract were collected in 10% buffered formalin for histopathologic evaluation. Sections were stained with routine hematoxylin and eosin stain.

Environmental sampling. Environmental sampling of the hatchery utilized Thioglycolate media, without dextrose (Remel™ Thermo Fisher Scientific™, Lenexa, KS). Surface swabs, chick fluff, and eggshell fragments were collected from the hatchers/hatch halls, chick separator room, chick processing room, and transport trucks. Cotton-tipped swabs, pre-moistened in BHI broth medium (Remel™ Thermo Fisher Scientific™, Lenexa, KS) were used to swab various surfaces within a 20x20 cm area to prevent overloading the media with clostridial spores. Surface swabs were immediately inoculated into thioglycolate tubes. Chick fluff or eggshell fragments were collected in Whirlpak® bags (Nasco®, Fort Atkinson, WI). All samples were transported on ice to the PRDL for further processing.

Laboratory procedures. Inoculated MacConkey and Blood plates and Tetrathionate tubes from postmortem examinations were incubated under aerobic conditions at 37° C for 24 hours. Thioglycolate tubes were incubated under anaerobic conditions at 37° C for 24 hours.

Eggshell pieces collected from the hatchery were manually broken down into small fragments in Whirlpak® bags. Chick fluff and eggshell samples were then inoculated into separate thioglycolate tubes. Tubes were incubated at 37°C in an anaerobic environment for 24 hours. Following incubation, all samples from postmortem examinations and environmental sampling were plated on anaerobic (CDC) Phenylethyl Alcohol Agar (PEA) plates (Remel™ Thermo Fisher Scientific™, Lenexa, KS), and incubated at 37°C in an anaerobic environment for 24 hours. If suspect hemolytic *C. perfringens* colonies were present following anaerobic incubation, two individual colonies were isolated and plated on separate Tryptic Soy Agar with sheep blood plates. One plate was incubated under anaerobic conditions, while the other was incubated under aerobic conditions, both at 37°C.

After 24 hours of incubation, colonies that grew under anaerobic conditions were further identified by VITEK® MALDI-TOF MS. Samples were prepared according to manufacturer recommendations. Each suspected culture was tested in duplicate to minimize the effect of variations due to potential mixed cultures. For instrument calibration, *Escherichia coli* ATCC 8739 was included with every 16 samples. Selected isolates were analyzed by 16S rRNA gene sequencing to validate VITEK® MALDI-TOF MS results and demonstrated a 99.8% genetic similarity to *C. perfringens*.

After *C. perfringens* detection, research-use only SARAMIS™ software (VITEK® MS Plus, bioMérieux, Inc., Durham, NC) was used to create a hierarchical clustering of *C. perfringens* isolates (SuperSpectra) obtained and to compare their mass spectrometric patterns. The SARAMIS™ module, which analyzes relative taxonomy, assessed genetic relatedness between bacterium isolates from live chickens collected at postmortem examination versus environmental samples. Following the software manufacturer recommendations, the target was established at 70 (14). Comparative isolates (i.e. chicken vs. environmental) with a spectrometric peak equal to or above the target (70) were considered identical strains (referred to as "duplicate" by SARAMIS™), while those above 65 were considered to belong to the same species. A subjective scale for interpretation was decided upon by the authors. Spectrometric peaks between two isolates equivalent to or greater than 70 was given a score of 3 (high degree of genetic relatedness), between 60.1 to 69.9 score 2 (moderate degree of genetic relatedness), and less than or equal to 60 score 1 (low degree of genetic relatedness).

RESULTS

Gross pathology. From the serosal surface, white, well-demarcated foci approximately 1 to 2 centimeters in length were evident on the duodenal loop. When evaluated from the mucosal surface, these foci correlated with white, plaque-like, ulcerative lesions. Sections of intestinal wall thinning, distension and congestion were also evident throughout the upper half of the gastrointestinal tract, predominantly in the duodenal loop. Additionally, birds had evidence of bacterial infection, characterized by fibrinocaseous pericarditis, peritonitis, and perihepatitis.

Bacteriology. Anaerobic culture was performed in two of three postmortem examinations. From both cases (live chickens, 5 and 6 days old), *C. perfringens* was isolated from intestinal lesion areas cultured. Heavy growth of *E. coli* was isolated from the liver, yolk sac, pericardium, or coelomic cavity, while *Salmonella spp* isolation attempts were negative in all three cases.

Histopathology. Histologically, multifocal, necro-ulcerative, heterophilic to lymphohistiocytic enteritis was diagnosed (Fig. 2A). At higher magnification, myriad, gram-positive, rod-shaped bacteria were noted within areas of ulceration and necrosis. The morphology of these bacteria is consistent with *C. perfringens.* No coccidial life stages were observed in affected sections of gastrointestinal tract.

Environmental sampling. Thirty-one samples were collected during environmental sampling. In total, 21 (68%) were positive for *C. perfringens.* This bacterium was identified in all areas of the hatchery sampled. Surface swabs yielded the highest number of positive samples (11/20), and the agent was isolated from nearly all samples of chick fluff (5/6) and all eggshell fragments (5/5) collected (Figure 1).

Mass spectrometric analysis of isolates. From all *C. perfringens* isolates obtained (n=27), 16 isolates representing each sampling category were included in the comparative analysis using SARAMIS™ software. Isolates demonstrated a 99.9% genetic relatedness to one another and were identified as "Superspectrum-Clostridium-perfringens-3". When comparing relatedness between isolates obtained from live, 5-day-old chickens from complex 1 and environmental isolates obtained during either hatchery sampling, identical strains (spectrometric peak ≥ 70) were identified from plenum fluff, spray cabinet swabs, box washer blower duct swabs, hatcher fluff, chick room swabs, hatcher eggshell, truck swabs, egg touches in setter, and egg touches in egg room 1, while the isolate from eggshells collected in the separator room was determined to be of the same species. For live, 6-day-old chickens from complex 2, identical environmental and live chick isolates were identified from plenum fluff, spray cabinet swabs, hatcher fluff, truck swabs, egg touches in setter, egg touches in egg room 1 and residue from used hatch trays. Isolates from box washer blower duct swabs, chick room swabs, hatcher eggshell, separator room eggshell, and egg touches in egg room 2 were identified as the same species as obtained from chicks. Figure 2 provides these results using the subjective scoring system designed by authors, where a score 3 indicates identical isolates.

DISCUSSION

This report describes a novel presentation of early clostridial enteritis, which included several aspects not typical of traditional necrotic or ulcerative enteritis. Firstly, clinical NE lesions, as described by Timbermont (7), were not always detected. Instead, the predominant lesion type observed were focal ulcerations. *C. perfringens* was isolated from all samples, despite its failure to consistently elicit gross lesions typical of clinical NE. Nonetheless, necrosis was histologically evident in affected sections of intestines, demonstrating some shared pathogenesis to clinical NE.

Secondly, the age of birds at the onset of disease differed from traditional NE presentations. Birds presented on the farm on or before 19 days of age. Typically, NE occurs in broiler flocks around three to

four weeks of age. This is often related to the amount of time required for coccidiosis cycling to occur, which is a major predisposing factor in the pathogenesis of NE (6, 7). In the current case, birds were infected, colonized and developed gross lesions as early as five or six days of age. Coccidiosis was not documented grossly or histologically in the submitted cases, further distinguishing this presentation from previous NE reports. As previously mentioned, prophylactic antibiotics were being given in the feed at both complexes and should have provided some protection to the development of this disease. Furthermore, rotation of anticoccidials products and vaccines was described by company veterinarians. Complete lack of oocysts at the time of necropsy supports good coccidiosis control. Given the wide range of affected farms, it is impossible to know what levels of stress could have been experienced by individual flocks. Although entirely speculative, it is possible that a strain of *C. perfringens* with increased virulence could be responsible for this disease syndrome given that a predisposing factor was not readily identified.

NetB toxins are the main virulence factors associated with the development of NE (15) and these toxins are exclusively produced by type A strains of *C. perfringens* (16). Molecular diagnostics for toxin typing and genomic analysis would be necessary to further evaluate the isolates from this case, but MALDI-TOF results demonstrate a 99.9% relatedness among all field and hatchery isolates collected. By using molecular typing techniques, other authors have reported isolation of identical clones throughout the entire flock from clinical cases of NE in the field (17). In contrast, healthy flocks seem to have a high degree of genetically diverse isolates, with as many as three distinct subtypes identified in a single bird (18). These reports support the concept that a strain of *C. perfringens* with increased virulence infected birds in this case, given the high degree of genetic relatedness among isolates and severity of lesions observed.

All eggshell fragments and all but one sample of fluff were positive for *C. perfringens*, suggesting this material may be an important reservoir for *C. perfringens* in the hatchery. In a similar study evaluating the incidence of *C. perfringens* in the hatchery, authors sampled eggshell fragments, chick fluff, and chick paper prior to and after use and reported the highest recovery of *C. perfringens* from chick fluff (9). In our study, analysis of spectrometric peaks indicated that environmental samples including chick fluff or surfaces in contact with chick fluff (i.e., plenum fluff, spray cabinet, hatcher fluff, box washer blower duct, truck surfaces, chick room surfaces) were often identical to isolates obtained from live chicks. In contrast, environmental samples where fluff was minimally or never present (i.e., separator room wall, egg touches in egg room 2, eggshell from separator room) had a lower relatedness to the isolates obtained from live chicks. These findings suggest environmental sampling of a hatchery for *Clostridium* species should focus on areas that collect fluff, particularly the hatchers and their associated plenum space.

As efforts are made to move away from the use of in-feed antibiotics in poultry production, it is likely that clostridial enteritis will become more prevalent in the industry (7). This case report demonstrated an epidemiologic link between hatchery contamination with *C. perfringens* and a novel presentation of enteritis in young broilers, involving necro-ulcerative enteritis in chickens less than three weeks of age associated with the isolation of *Clostridium perfringens*. While Clostridium is not often considered an important hatchery contaminant, this case report supports the concept that future efforts at hatchery monitoring should include this pathogen, particularly if early enteritis is being experienced on the farm.

ACKNOWLEDGEMENTS

The authors would like to thank Zoetis, Inc. and Huvepharma for the financial support of this project.

REFERENCES

1. Immerseel FV, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. *Avian Pathol.* 33: 537-549; 2004.

2. ITIS. Integrated Taxonomic Information System – Report. *Clostridium Perfringens.* Taxonomic Serial No.: 555646. Generated: Wednesday, September 8, 2021. https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=555646#null

3. Boulianne M. Clostridial Diseases: Introduction. In: Swayne DE, editor. *Diseases of poultry.* 14th ed. Hoboken (NJ): Wiley-Blackwell. P. 966; 2020.

4. Martel A, Devriese LA, Cauwerts K, De Gussem K, Decostere A, Haesebrouck F. Susceptibility of *Clostridium perfringens* strains from broiler chickens to antibiotics and anticoccidials. *Avian Pathol.* 33: 3-7; 2004.

5. Cooper KK, Songer JG. Necrotic enteritis in chickens: a paradigm of enteric infection by *Clostridium perfringens* type A. *Anaerobe* 15: 55-60; 2009.

6. Immerseel F V, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the

pathogenesis of necrotic enteritis in chickens. *Trends Microbiol.* 17: 32-36; 2009.

7. Timbermont L, Haesebrouck F, Ducatelle R, Immerseel FV. Necrotic enteritis in broilers: an updated review on the pathogenesis. *Avian Pathol.* 40: 341-347; 2011.

8. Craven SE, Stern NJ, Bailey JS, Cox NA. Incidence of *Clostridium perfringens* in broiler chickens and their environment during production and processing. *Avian Dis.* 45: 887-896; 2001.

9. Craven SE, Cox NA, Stern NJ, Maudlin JM. Prevalence of *Clostridium perfringens* in commercial broiler hatcheries. *Avian Dis.* 45: 1050-1053; 2001.

10. Udhayavel S, Ramasamy GT, Gowthaman V, Malmarugan S, Senthilvel K. Occurrence of *Clostridium perfringens* Contamination in poultry feed ingredients: isolation, identification, and its antibiotic sensitivity pattern. *Anim. Nutr.* 3: 309-312; 2017.

11. Shane SM, Koetting DG, Harrington KS. The occurrence of *Clostridium perfringens* in the intestine of chicks. *Avian Dis.* 28: 1120-1124; 1984.

12. Craven SE, Cox NA, Bailey JS, Cosby DE. Incidence and tracking of *Clostridium perfringens* through an integrated broiler chicken operation. *Avian Dis.* 47: 707-711; 2003.

13. Opengart K, Boulianne M. Clostridial Diseases: Necrotic Enteritis. In: Swayne DE, editor. *Diseases of poultry.* 14th ed. Hoboken (NJ): Wiley-Blackwell. P. 972-976; 2020.

14. VITEK® MS. Software version 3.1. Knowledge Base Version 2.2. Customer training Manual. SARAMIS™ software: SuperSpectra creation & other Research functionalities. BIOMERIEUX University. 2020. BioMerieux, Inc. Durham, North Carolina.

15. Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Di Rubbo A, Rood JI, Moore RJ. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens. PLOS Pathogens* 4(2); 2008. *https://doi.org/10.1371/journal.ppat.0040026*

16. Keyburn AL, Bannam TL, Moore RJ, Rood JI. NetB, a pore-forming toxin from necrotic enteritis strains of *Clostridium perfringens*. *Toxins* 2: 1913-1927; 2010.

17. Nauerby B, Pedersen K, Madsen M. Analysis by pulsed-field gel electrophoresis of the genetic diversity among *Clostridium perfringens* isolates from chickens. *Vet Microbiol.* 94: 257-266; 2003.

18. Engström BE, Fermér C, Lindberg A, Saarinen E, Båverud V, Gunnarsson A. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. *Vet Microbiol.* 94: 225-235; 2003.

Figure 1. Distribution of positive *Clostridium perfringens* samples based on sample type.

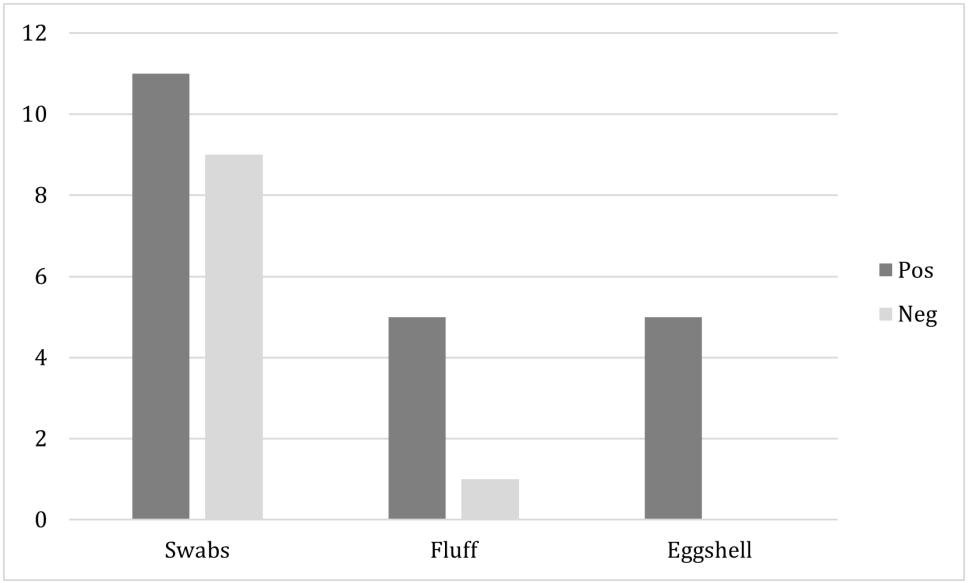
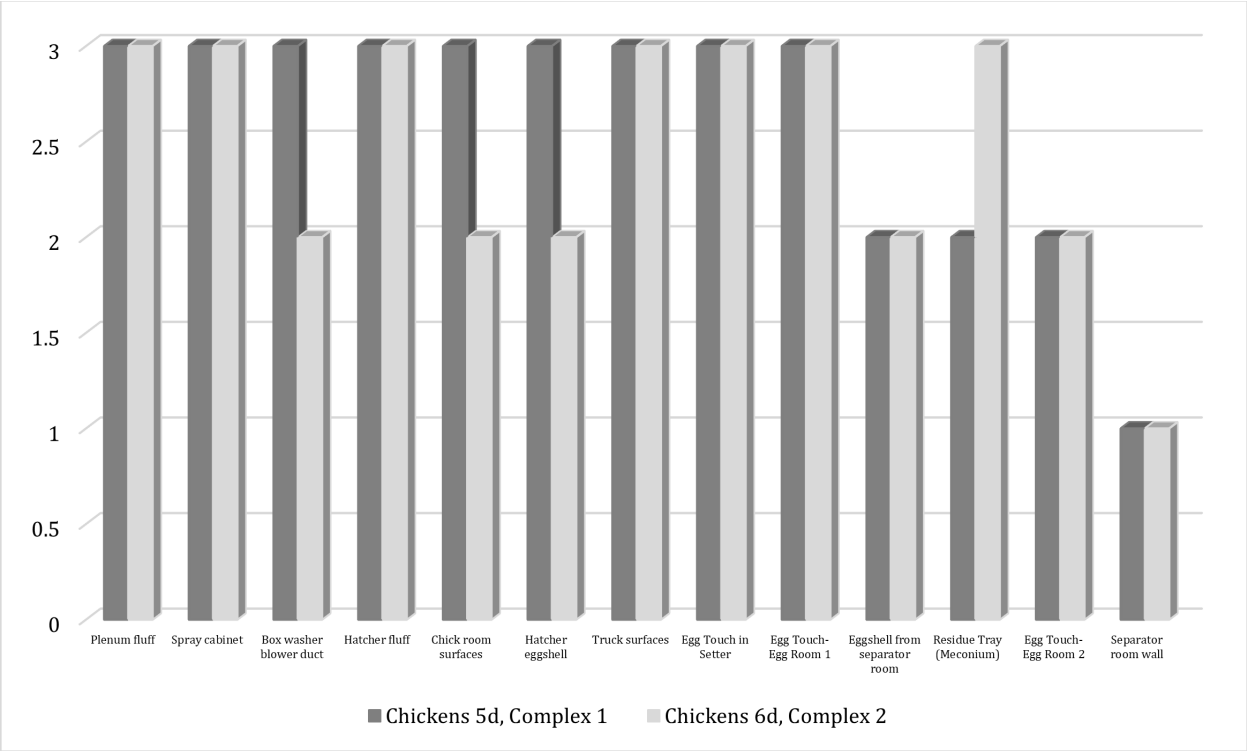


Figure 2. Research-use only SARAMIS™ taxonomy results of *C. perfringens* isolates, comparing those obtained from live chicken samples (5 vs. 6 days of age) to those obtained from environmental sampling at the hatchery. Analysis made based on subjective scoring where score 3 indicates isolates are highly genetically related (identical with a peak ≥ 70), score 2 indicates a moderate degree of relatedness (same species with a peak between 60.1 to 69.9), while score 1 indicates the lowest degree of genetic relatedness (peak ≤ 60).



CASE REPORT: THAT'S NOT SUPPOSED TO HAPPEN AFTER VACCINATION, RIGHT?

S. Throne[A], M. McConnell[A], F. Myers[A], R. Moore[B], and M. Ficken[C]

[A]Simmons Foods, Inc., Siloam Springs, AR 72761
[B]Veterinary Diagnostic Laboratory, Fayetteville, AR 72703
[C]Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX 77843

SUMMARY

Hepatopathy is a curious syndrome induced by the use of autogenous *Salmonella* bacterin. This case report will describe multiple cases of hepatopathy that happened over the course of several months. The first case presented as ruptured livers but diagnosis was confounded by lack of history, mycotoxin issues in the complex and piling issues with the flock. Further investigation involved making observations with company and contract vaccination crews to evaluate bird and vaccine handling at the time of field vaccination. Improvements to vaccine injection and warming temperatures were made and it appeared that these caused improvements and the problem was corrected until it reoccurred five weeks later. In the meantime, histopathology samples from the first case revealed chronic amyloidosis. It wasn't until the issue occurred on another farm that we were able to determine the causative issue. During this case report, we will present clinical signs, production records and pathology reports, as well as discussing our investigative process. We determined that the issue was linked to a new serial of vaccine when used with one particular breed, which could be exacerbated with vaccination technique.

CLINICAL SIGNS AND NECROPSY FINDINGS

Multiple cases presented over the course of two months with common traits noted throughout. Cases all presented five to eight days after the 2nd vaccine handling at approximately 18 weeks of age. Most flocks presented with an acute increase in mortality. Large volume of hemorrhagic fluid was present in the coelomic cavity. Livers were enlarged, swollen, pale. Some livers were noted to be very firm while others were quite friable. Ruptured livers were noted in many of the birds due to their friable nature. Injection site reactions were prominent in the thigh muscle and lower abdominal cavity. Additional findings included: pericarditis and/or opacity to the pericardium, fluid consolidation of the lungs, pale kidneys and/or spleen, and enlargement of spleen.

HISTOPATHOLOGY

Chronic, severe pyogranulomatous myositis and cellulitis with foreign material was noted in the thigh and the abdominal cavity, which was consistent with vaccine reaction due to vaccine being given intramuscularly. In the liver, acute multifocal coalescing necrosis and hemorrhage was noted along with chronic diffuse amyloidosis. The amyloidosis was characterized as mild, marked and/or severe depending on the case presentation. In some sections, intralesional bacterial were also seen; however, this may have been an incidental finding. Within the spleen, marked amyloidosis and lymphoid depletion was noted. The kidneys consistently had no significant lesions.

The severity of the amyloidosis noted in the samples was considered to be a sequela to chronic inflammation or vaccination. The acute hemorrhage and necrosis were a response to the bacterial infection or compromised hepatic parenchyma. The lesions with the thigh muscle was consistent with vaccine induced granuloma.

As a comparison, birds were observed and sampled that were a different breed and vaccinated by a different crew. Clinicals signs, necropsy or histopathology were not noted in any of those birds as compared to our hepatopathy affected birds.

DISCUSSION/CONCLUSION

The first steps of our investigation centered on vaccination technique and vaccine handling due to the granulomas. Placement of the vaccine was emphasized as well as making minor improvements on technique. Evaluation of vaccine handling found that viral vaccines and bacterial bacterins were being warmed in the same coolers to the same temperatures. The use of separate vaccine coolers was instituted so that different temperatures could be achieved for each of the vaccine types. Making these changes improved the situation, but did not solve the problem entirely and further investigation was warranted.

A spreadsheet was developed to track all pullet flocks by house and included male and female breeds,

age, date of vaccination, vaccination crew, vaccine type, manufacturer, serial number and clinical signs/necropsy findings. This allowed a pattern to emerge. The severe clinical presentation and subsequent pathology was only seen after a new autogenous serial was used in production and then only typically when used with one female breed and to a lesser extent another female breed line from within the same company. This autogenous bacterin contained the same four *Salmonella* isolates that had been used in the previous bacterin and contained the same adjuvant.

The use of that bacterin serial was immediately stopped in those two female breeds and a commercial bacterin used in its place. Since this change, no additional cases have been observed.

REFERENCES

1. Hemorrhagic Hepatopathy in Broiler Breeder Pullets: Gross and Microscopic Pathology and Factors Associated with Incidence. Linnea M Tracy, Eric Shepherd, Monique Franca, Susan Williams, Karen Grogan, Jenny Nicholds, Katharine Shamoun, and ChangHee Lee. Avian Diseases. 65:269-280, 2021.

ISOLATION, SPECIATION, AND ANTICOCCIDAL SENSITIVITY OF *EIMERIA* SPP. RECOVERED FROM WILD TURKEY FECES

C. Trujillo[A], D. Graham[A], A. Ashcraft[A], R. Senas Cuesta[A], M. Coles[A], C. Selby[A], A. Forga[A], G. Tellez-Isaias[A], C. Vuong[A],
J. Barta[B], and B. Hargis[A]

[A]Department of Poultry Science, University of Arkansas Division of Agriculture, Fayetteville 72701, USA
[B]Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada

SUMMARY

From 2018-2020, wild turkey fecal samples from across the Eastern two thirds of the United States were collected from areas where commercial turkey production is not common. To date, we have received more than 100 wild turkey fecal samples. We hypothesized that wild turkey fecal samples would contain *Eimeria* spp. possessing drug sensitivity. Samples containing *Eimeria* spp. oocysts were amplified *in vivo*. If propagation was successful, the samples were PCR-speciated and subjected to turkey anticoccidial sensitivity (TACS) testing for major representatives of both the ionophore and chemical categories of anticoccidial drugs. The purpose of this study was to obtain the major *Eimeria* spp. important for commercial turkey production that had sensitivity to monensin, zoalene, and amprolium. Single oocyst-derived stocks would be used in future studies to evaluate efficacy of wild turkey *Eimeria* spp. as vaccine candidates to control coccidiosis in commercial turkey flocks.

INTRODUCTION

Coccidiosis is a caused by an Apicomplexan protozoan of the genus *Eimeria* that causes significant economic losses for the commercial poultry industry (1,2). Historically, anticoccidial drugs, either ionophores or chemicals, have been used to control coccidiosis in commercial poultry operations. Coccidial resistance to ionophores develops slowly, and rotating programs with different ionophores, as well as a restricted number of effective chemical coccidiostats, has slowed the formation of multi-drug resistant coccidial species. However, due to consumer-driven markets, increased regulatory environments, and evidence of multi-drug resistance, alternative strategies to control coccidiosis are needed (1,2). Drug-sensitive live coccidiosis vaccines have been used for commercial broiler and layer production, both to control disease and to renew sensitivity to available drugs for control, but the turkey industry is limited to commercially available vaccines with limited species coverage (1,2,3).

Since *Eimeria* spp. relevant to commercial turkeys also circulate within wild turkey populations (4,5), we hypothesized that wild turkey fecal samples collected from geographical regions absent of commercial turkey operations would contain drug-sensitive *Eimeria* species. These drug-sensitive *Eimeria* spp. could be used to generate a multi-species live vaccine as an alternative vaccination program for the commercial turkey industry. This goal of the present study was to isolate drug-sensitive strains of *E. adenoides, E. gallopavonis, E. meleagrimitis, E. meleagridis,* and *E. dispersa* from wild turkey feces.

MATERIALS AND METHODS

Recovery of wild turkey *Eimeria* spp. Feces was collected by individuals and state agencies across the United States. Samples were placed on wet ice packs and shipped to the UADA Poultry Health Laboratory for further processing. Light microscopy was used for initial detection of oocysts. A subset of the fecal sample was diluted with saturated salt solution and evaluated using a McMaster counting chamber. The chamber was examined for the presence/absence of oocysts and recorded as oocyst-positive or oocyst-negative. Oocyst-positive samples were partially processed, and oocysts were sporulated as previously described (6,7). No further processing was done for oocyst-negative samples. Due to the low number of oocysts present in the majority of fecal samples obtained, amplification/propagation and sporulation was conducted to obtain a fresh oocyst stock was required before moving forward with TACS.

Speciation. DNA extraction and PCR identification were conducted using procedures modified from El-Sherry *et al.* (7). Following primary PCR for amplification of mitochondrial cytochrome oxidase subunit I (COI) gene, each purified primary PCR product was used as a template for secondary PCR amplification using species-specific primers for

E. adenoides, E. gallopavonis, E. dispersa, E. innocua, E. meleagridis, and *E. meleagrimitis*. All positive-secondary PCR products were sent to Eton Bioscience (Eton Bioscience, Inc., Raleigh, NC, USA) for sequencing to confirm PCR results.

Turkey anticoccidial sensitivity test (TACS). Anticoccidial sensitivity was calculated based on the reduction of oocyst shedding in the medicated, challenged group compared to the non-medicated, challenged control group (CC). For TACS, there were six turkeys utilized per sample/evaluation (n=3 for CC, n=3 for medicated). Anticoccidial medication, either monensin (75g/ton), zoalene (454g/ton), or amprolium (0.024%/drinking water), began two days prior to challenge. At nine or 10 days-of-age, approximately 100 sporulated oocysts (aged <6 months) per mL was orally administered to respective CC and medicated group. Feces was collected from day five to nine days post-challenge. Based on total oocyst output between CC and medicated groups, samples were deemed resistant (<30% reduction), partially sensitive (31-79% reduction), or sensitive (>79% reduction) to monensin, zoalene, or amprolium.

Single oocyst-derived stocks. To obtain pure stocks of individual drug-sensitive *Eimeria* spp., one sporulated oocyst was isolated from a mixed species sample and was administered orally to one turkey between 12-14 days-of-age. The process was conducted as described by Remmler and McGregor (8) with similar modifications described by El-Sherry *et al.* (7). Turkeys were housed in individual cages. Feces was collected from day 4-10 post-challenge. Fecal samples were partially processed, concentrated as appropriate, and sporulated as previously described. These stocks were subjected to amprolium TACS.

RESULTS AND DISCUSSION

A total of 106 fecal samples were collected from wild turkeys across Arkansas (AR), Louisiana (LA), Texas (TX), Delaware (DE), Pennsylvania (PA), and Maine (ME). *Eimeria* spp. oocysts were detected in 78.3% (83/106) of the samples, including 100% of the samples recovered from DE (36/36), PA (38/38), or ME (9/9). This suggests that *Eimeria* cycling is ongoing in wild turkey populations in these areas. Although fecal samples from wild turkeys located in regions of AR, TX, and LA were received, the quality of the samples was poor and could not be evaluated.

Due to the low concentration of *Eimeria* spp. oocysts in the majority of the samples obtained from DE, PA, and ME, an amplification step was required to obtain a fresh stock of oocysts for TACS testing and speciation. Viable sporulated oocysts were recovered after the amplification and sporulation steps for 49.4% (41/83) of the samples. Of the 41 samples, there were 22 DE, 11 PA, and 8 ME samples subjected to monensin TACS testing. There were 11/41 (26.8%) sensitive, 22/41 (53.7%) had reduced sensitivity, and 8/41 (19.5%) were resistant to monensin (Table 1). There were 20/41 (48.8%) samples with a sensitivity threshold >70% for monensin that were subjected to zoalene TACS testing. Of the 20 samples, 9/20 (45%) were sensitive, 8/20 (40%) had reduced sensitivity, and 3/20 (15%) were sensitive to zoalene (Table 1).

Speciation results for 35/41 samples subjected to monensin and/or zoalene TACS is shown in Table 2. More than one *Eimeria* spp. was detected in 25/35 (71.4%) of the samples, whereas 10/35 (28.6%) samples contained only *E. meleagrimitis*. Top candidates were selected based on sensitivity to monensin and zoalene and used to generate single oocyst-derived stocks. An amplification step was required for candidate stocks to obtain enough oocysts to confirm recovery of a single *Eimeria* spp. (PCR and sequence confirmation) and to re-confirm sensitivity to monensin, zoamix, and amprolium. Seven stocks were generated and 7/7 (100%) were confirmed to be sensitive to amprolium (Table 1). Currently, we have single oocyst-derived stocks that are sensitive to monensin, zoamix, and amprolium for the following *Eimeria* spp.: *E. meleagrimitis, E. meleagridis, E. dispersa, E. gallopavonis*. Studies to obtain a single oocyst-derived stock for *E. adenoides* are ongoing. Immunogenicity studies are also underway.

(The full-length article will be submitted to a relevant journal.)

ACKNOWLEDGEMENTS

This research was funded by USDA Animal Health Funds (FY21).

REFERENCES

1. Chapman, HD. Coccidiosis in the turkey. Avian Pathology 37(3): 205-223; 2008.
2. Chapman HD, Jeffers TK. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. International Journal for Parasitology: Drugs and Drug Resistance 4(3): 214-217; 2014.
3. Chapman HD, Roberts B, Shirley MW, Williams RB. Guidelines for evaluating the efficacy and safety of live anticoccidial vaccines, and obtaining approval for their use in chickens and turkeys, Avian Pathology, 34:4, 279-290; 2005.
4. Ruff MD, Schorr RL, Davidson WR, Nettles VF. Prevalence and identity of coccidian in

pen-raised wild turkeys. Journal of Wildlife Diseases. 24(4):711-714; 1988.

5. MacDonald AM, Jardine CM, Rejman E, Barta JR, Bowman J, Cai HY, Susta L, Nemeth NM. High prevalence of Mycoplasma and Eimeria species in free-ranging eastern wild turkeys (Meleagris gallopavo silvestris) in Ontario, Canada. Journal of wildlife diseases, 55(1):54-63; 2009.

6. Reid WM, and Long PL. A diagnostic chart for nine species of fowl coccidia. The University of Georgia College of Agriculture Experimental Stations. Research report, 335:1-24; 1979.

7. El-Sherry S, Ogedengbe ME, Hafeez MA, Barta J. Divergent nuclear 18S rDNA paralogs in a turkey coccidium, Eimeria meleagrimitis, complicate molecular systematics and identification. International Journal for Parasitolog, 43(8): 679-685; 2013.

8. Remmler O, McGregor JK. A method to facilitate isolation of single coccidial oocysts. The Journal of Parasitology, 50:294; 1964

Table 1. TACS (monensin, zoalene, and amprolium) testing for select wild turkey *Eimeria* spp. recovered Delaware, Pennsylvania, and Maine.

	Monensin[1]			Zoalene			Amprolium[2]		
Location	R	RS	S	R	RS	S	R	RS	S
DE	6/22	11/22	5/22	6/10	4/10	0/10	0/3	0/3	3/3
PA	1/11	7/11	3/11	2/4	2/4	0/4	-	-	-
ME	1/8	4/8	3/8	1/6	2/6	3/6	0/4	0/4	4/4
Total	8/41	22/41	11/41	9/20	8/20	3/20	0/7	0/7	7/7

[1] Samples with >70% sensitivity to monensin were evaluated for zoalene sensitivity

[2] Single oocyst-derived stocks (n=7) were generated from select mixed species samples and evaluated for amprolium sensitivity

Classification: <30% reduction, R=resistant; 31-79% reduction, RS=reduced sensitivity; >79% reduction, S=sensitive

Table 2. Speciation of wild turkey *Eimeria* spp. recovered Delaware, Pennsylvania, and Maine.

Location	*E. adenoeides*	*E. dispersa*	*E. gallopavonis*	*E. innocua*	*E. meleagridis*	*E. meleagrimitis*
DE	10/17	10/17	2/17	3/17	2/17	15/17
PA	4/10	7/10	1/10	5/10	1/10	8/10
ME	1/8	2/8	0/8	0/8	1/8	6/8
Total	15/35	19/35	3/35	8/35	4/35	30/35

FOCAL DUODENAL NECROSIS: IDENTIFICATION OF GRAM-NEGATIVE BACTERIA IN INTESTINAL LESIONS

Y. Tsai[A], M. Franca[A], N. Barbieri[A], A. Camus[B], and C. Logue[A]

[A]Department of Population Health, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602
[B]Department of Pathology, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602

INTRODUCTION

Focal duodenal necrosis (FDN) is an intestinal disease of table egg layers. It was first described in a cage-free layer flock at the Pennsylvania State University in 1996. Since then, the disease has been diagnosed in most states in the US and Europe (4). FDN is considered one of the top five concerning diseases in the table egg layer industry according to the Association of Veterinarians in Egg Production (7). It has been detected in all major genetic lines of laying chickens raised in different management systems; cage, cage-free and organic (21). The economic impact of FDN can result in decreased egg case weight and a reduction in egg production.

Despite the first description of FDN in 1996, the etiology has not been identified. Some studies found FDN is associated with *Clostridium* species. One study found a higher frequency of *Clostridium colinum* signature gene markers in duodenal samples from affected chickens (16). In a 2016 study, research reported the association of beta2-positive *C. perfringens* type A with FDN in egg layers in the United States (5). Moreover, the epidemiological profile of FDN-affected flocks was established and the results indicated several management and dietary practices in FDN affected flocks that may induce predisposing factors that cause enteric diseases (23). In 2018, the same research group tried to reproduce FDN by using different *Clostridium perfringens* isolates with different feed ingredients. Although it failed to reproduce characteristic lesions of FDN, it did cause enteritis in the challenge group (24).

To figure out what might be the missing element associated with the etiology of FDN, we turn to another target. We frequently find Gram-negative long rod-shaped bacteria within the FDN lesion, but the genus and species of this bacterium is unknown. Therefore, in this research, we aim to identify the Gram-negative bacteria commonly found in association with FDN lesions. Our central hypothesis is that a Gram- negative bacterium plays an important role in the pathogenesis of FDN. We used Laser Capture Microdissection followed by Sequencing of the 16S rRNA gene as a targeted molecular approach to identify the bacteria. Also, we isolated *Escherichia coli* from FDN lesions and used PCR to identify isolates and to determine their virulence genes.

MATERIALS AND METHODS

Ethanol-fixed sample collection. A total of 59 ethanol-fixed duodenum samples from 8 different farms located in Idaho, Indiana, Iowa, Nebraska, and Ohio were examined. Samples were collected between September 14th, 2020 and March 14th, 2021. The duodenum samples were fixed in 70% ethanol-filled jars and shipped to the Poultry Diagnostic and Research Center. Eight negative control samples from 3 different control (FDN-negative) layer farms were also included in the analysis.

Histopathology. Every sample was grossly examined for FDN lesions (focal to multifocal erosions in the duodenum) then trimmed, embedded in paraffin, sectioned at 4 µm, mounted on glass slides, and stained with hematoxylin and eosin (H&E). All slides were examined by bright field microscopy to confirm the presence of FDN microscopic lesions.

Laser capture microdissection. Histologic sections were prepared on MMI nuclease-free membrane slides, and the areas with long rod-shaped bacteria were collected using an MMI CellCut Plus® microdissection system (Molecular Machines & Industries, Eching, Munich, Germany). The collected tissue was attached to the lid of MMI collection tube. For negative control samples, 25 random mucosal layer and exudate areas were excised from each slide.

DNA extraction. Genomic DNA was isolated from dissected tissue samples using the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's instructions. The purified DNA was eluted from the spin filter using 100uL of solution C6 and stored at -20°C until PCR amplification.

PacBio sequencing. The 16S rRNA gene PCR primers 27Fwith barcode on the forward primer were used in a 35-cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. The PCR pool was then purified using Ampure PB beads (Pacific Biosciences).

The SMRTbell libraries (Pacific Biosciences) were prepared following the manufacturer's user guide and sequencing performed by MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on the PacBio Sequel following the manufacturer's guidelines. After completion of initial DNA sequencing, each library undergoes a secondary analysis, Circular Consensus Sequencing, using PacBio's CCS algorithm. Sequence data was then processed using the MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Final OTUs were taxonomically classified using BLASTn against a curated database derived from NCBI (www.ncbi.nlm.nih.gov) and compiled for each taxonomic level into both "counts" and "percentage" files. Counts files contain the actual number of sequences while the percent files contain the relative abundance of each OTU.

Fresh sample collection. A total of 20 fresh duodenal samples from layer farms with history of FDN were examined macroscopically to confirm the presence of FDN lesions. The lesions were trimmed to size to reduce the amount of redundant tissue, split into equal parts and placed in 200-300 uL of Buffered Peptone water with incubation at 37°C for 18-24 h both aerobically and anaerobically. Following incubation, the enrichment broths were struck to 5% sheep blood agar plates (Remel) and Tryptone Soy Agar (TSA, Difco) with incubation of the plates at 37°C for 18-24 h both aerobically and anaerobically. Following incubation, suspect isolates were selected for further characterization.

PCR analysis. Suspect *E. coli* isolates from lesion samples were confirmed using 16S PCR using the forward primer (UAL1939-187) and reverse primer (UAL2105-187). Isolates were also subject to analysis using Path PCR primers and protocols for genes *fimH, dsbA, eaeH, fepC, ratA, colV, usp* and *irp2* using multiplex PCR (2, 11). The PCR primers and protocol for colicin detection and Microcin were described by Gordon et al, 2006 (8). Other IBD virulence determinants (*ial, aer, pap* and *cnf1*) were tested using primers and conditions described by Birosova et al and López-Saucedo et al (1, 12). All amplification products were analyzed by gel electrophoresis, followed by staining in 0.25% Ethidium Bromide solution (Sigma Aldrich. St. Louis, MO) for 20 min and viewed under UV light using Omega Lum G imager (Aplegen, San Francisco, CA).

RESULTS

From a total of 59 ethanol-fixed duodenal samples, 43 were diagnosed with FDN by histopathology. Macroscopically, there were focal to multifocal superficial erosions in the duodenal mucosa. Microscopically, lesions were characterized by heterophilic and lymphoplasmacytic enteritis with mild enterocyte necrosis, and the presence of luminal fibrinonecrotic exudate with variable numbers of long rod-shaped bacteria observed within lesions. Using laser capture microdissection, samples of the lesions were cut and sent to Mr. DNA (city and state) where DNA extraction, 16S RNA PCR and sequencing analysis were carried out. The sequencing results, in the relative species abundance analysis, failed to show any consistent bacterial species present among all samples analyzed. In the relative phylum abundance analysis, we found Proteobacteria and Actinobacteria more abundant in FDN samples than in control FDN-negative samples (Figure 1), indicating intestinal dysbiosis. In contrast, phylum Firmicutes was found to be more abundant in control samples. These results suggest significant differences in the duodenal microbiota between layers with FDN and healthy birds. Similar dysbiotic gut microbiota is found in inflammatory bowel disease (IBD) including Crohn's disease (CD) as well as ulcerative colitis (UC) in humans, dogs, and other mammalian species (10, 15, 19). In a paper based on 16S rRNA gene libraries of IBD-affected dogs, a similar finding noted the absence of a single bacterial etiology, instead, phylotype level differences were observed between positive and negative controls (19). In humans, similar research found a depletion of Firmicutes in CD and UC patients compared to healthy controls (10). Another research that used FISH analysis of the mucosa-adherent bacteria in the ileum of CD patients also found a depletion of Firmicutes (17). Similarly, we also found depletion of Firmicutes in FDN samples compared to negative controls. Proteobacteria was more prevalent in FDN-affected birds than healthy controls. In human CD patients, Proteobacteria are commonly overrepresented (6, 14, 20). Not only in humans, but cats with IBD also show increased abundance of Proteobacteria in the inflamed intestine (9). These patterns of microbiota differences between FDN and control samples suggest FDN is associated with a dysbiotic gut.

Ten out of 20 fresh samples had FDN lesions upon gross observation. These samples were subjected to enrichment for bacterial detection and 30 aerobic and 17 anaerobic bacterial isolates were cultured from TSA and blood agar plates. To understand if these isolates were *E. coli* or not, an *E. coli* 16S PCR was carried out. PCR identified 38 isolates as *E. coli.* Sanger sequencing, in remaining 8 isolates, identified two isolates as *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, while six isolates were not identified. Therefore, we only analyzed 38 *E. coli* isolates for virulence genes by PCR. The Patho genes PCR showed 27/47 (53.8%) *E. coli* isolates had more

than 4 Patho genes, which indicated Avian pathogenic *Escherichia coli* (APEC). APEC can be found in the intestinal microflora of healthy birds; however most APEC are associated with extra intestinal disease which can be a secondary infection as a result of environmental or other predisposing factors (3). Among 9 APEC Patho genes detected, *ompT*, *hlyF* were the most prevalent (66.7%) followed by *iroN*, and *iss* (53.8%). These genes are considered important in the pathogenesis of APEC (18). In order to know whether the *E. coli* isolates from FDN are similar to *E. coli* associated with IBD, we screened for IBD-related virulence genes using multiplex PCR (Figure 2). The results found that some genes were very prevalent, for example *fimH* (97.4%), *dsbA* (94.9%), *eaeH* (92.3%), *fepC* (87.2%), and mH47 (94.9%). The primers for IBD multiplex PCR used in this study are a custom panel designed by our research group which suggest these isolates harbor virulence genes related to IBD. In addition, virulence genes and bacteriocin genes including colicins and microcins were also detected. In one study by Lenka Micenkova et al, 2018, analysis of 178 *E. coli* isolates from IBD patients for bacteriocin production revealed 30 bacteriocin and 22 virulence determinants (13). We chose the top three or four most prevalent genes from each group for our analysis. PCR results found microcin gene with a prevalence rate of 48.7%-94.9%, the colicins were detected at a rate of 15.4%-46.2%. Overall, *E. coli* from FDN lesions appear to possess multiple virulence genes associated with IBD. Although a comparative genomic study of *E. coli* isolates from IBD patients did not identify IBD-specific *E. coli* genes, the results from our research may still provide the insights that there might be some correlation between *E. coli* isolates from FDN lesions and those from IBD (22).

In conclusion, no single bacterial species was consistently associated with FDN, and LCM followed by microbiota sequencing revealed gut dysbiosis in FDN lesions. *E. coli* isolates from FDN lesions contain multiple virulence genes that have been identified in *E. coli* isolated from IBD. These findings suggest that these *E. coli* strains might play a role in the pathogenesis of FDN but further analysis of these isolates by pathogenicity studies to try to reproduce the disease is warranted. Therefore, FDN appears to be a multifactorial inflammatory intestinal disease associated with dysbiosis and the proliferation of Gram-negative bacteria that may contribute to the pathogenesis of this disease.

REFERENCES

1. Bírošová, E., L. Siegfried, M. Kmet'ova, A. Makara, A. Ostró, A. Grešová, P. Urdzík, A. Liptáková, M. Molokáčová, and R. Bártl. Detection of virulence factors in α-haemolytic Escherichia coli strains isolated from various clinical materials. Clinical microbiology and infection 10:569-573. 2004.

2. De Oliveira, A. L., D. M. Newman, Y. Sato, A. Noel, B. Rauk, L. K. Nolan, N. L. Barbieri, and C. M. Logue. Characterization of avian pathogenic Escherichia coli (APEC) associated with turkey Cellulitis in Iowa. Frontiers in Veterinary Science 7:380. 2020.

3. Dho-Moulin, M., and J. M. Fairbrother. Avian pathogenic Escherichia coli (APEC). Veterinary research 30:299-316. 1999.

4. Dunn, P. Layer: Pullet Regional Disease of Notheast US. In: Midwest Poultry Federation Convention. St. Paul, Minnesota, United States. 2015.

5. França, M., M. Barrios, L. Stabler, G. Zavala, H. Shivaprasad, M. Lee, A. Villegas, and F. A. Uzal. Association of beta2-positive Clostridium perfringens type A with focal duodenal necrosis in egg-laying chickens in the United States. Avian diseases 60:43-49. 2016.

6. Frank, D. N., A. L. S. Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the national academy of sciences 104:13780-13785. 2007.

7. Gingerich, E. Table Egg Industry Report In: Report of the USAHA Committee on Poultry and Other Avian Species. In., Kansas City, MO. 2018.

8. Gordon, D. M., and C. L. O'Brien. Bacteriocin diversity and the frequency of multiple bacteriocin production in Escherichia coli. Microbiology 152:3239-3244. 2006.

9. Janeczko, S., D. Atwater, E. Bogel, A. Greiter-Wilke, A. Gerold, M. Baumgart, H. Bender, P. McDonough, S. McDonough, and R. Goldstein. The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. Veterinary microbiology 128:178-193. 2008.

10. Kotlowski, R., C. N. Bernstein, S. Sepehri, and D. O. Krause. High prevalence of Escherichia coli belonging to the B2+ D phylogenetic group in inflammatory bowel disease. Gut 56:669-675. 2007.

11. Logue, C. M., Y. Wannemuehler, B. A. Nicholson, C. Doetkott, N. L. Barbieri, and L. K. Nolan. Comparative Analysis of Phylogenetic Assignment of Human and Avian ExPEC and Fecal Commensal Escherichia coli Using the (Previous and Revised) Clermont Phylogenetic Typing Methods and its Impact on Avian Pathogenic Escherichia coli (APEC) Classification. Frontiers in Microbiology 8. 2017.

12. López-Saucedo, C., J. F. Cerna, N. Villegas-

Sepulveda, R. Thompson, F. R. Velazquez, J. Torres, P. I. Tarr, and T. Estrada-García. Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic Escherichia coli. Emerging infectious diseases 9:127. 2003.

13. Micenková, L., L. Frankovičová, I. Jaborníková, J. Bosák, P. Dítě, J. Šmarda, M. Vrba, A. Ševčíková, M. Kmeťová, and D. Šmajs. Escherichia coli isolates from patients with inflammatory bowel disease: ExPEC virulence-and colicin-determinants are more frequent compared to healthy controls. International Journal of Medical Microbiology 308:498-504. 2018.

14. Packey, C. D., and R. B. Sartor. Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Current opinion in infectious diseases 22:292. 2009.

15. Prindiville, T., M. Cantrell, and K. H. Wilson. Ribosomal DNA sequence analysis of mucosa-associated bacteria in Crohn's disease. Inflammatory bowel diseases 10:824-833. 2004.

16. Siragusa, G., T. Baltzley, S. Dunham, F. Lago, and T. Rehberger. Molecular pathogenesis markers of focal duodenal necrosis in layer hens. In: 2008 Northeastern Conference on Avian Diseases (NECAD) proceedings, scientific meeting section. In: 80th Northeastern Conference on Avian Diseases. 2008.

17. Sokol, H., B. Pigneur, L. Watterlot, O. Lakhdari, L. G. Bermúdez-Humarán, J.-J. Gratadoux, S. Blugeon, C. Bridonneau, J.-P. Furet, and G. Corthier. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proceedings of the National Academy of Sciences 105:16731-16736. 2008.

18. Subedi, M., H. Luitel, B. Devkota, R. K. Bhattarai, S. Phuyal, P. Panthi, A. Shrestha, and D. K. Chaudhary. Antibiotic resistance pattern and virulence genes content in avian pathogenic Escherichia coli (APEC) from broiler chickens in Chitwan, Nepal. BMC veterinary research 14:1-6. 2018.

19. Suchodolski, J. S., P. G. Xenoulis, C. G. Paddock, J. M. Steiner, and A. E. Jergens. Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. Veterinary microbiology 142:394-400. 2010.

20. Swidsinski, A., A. Ladhoff, A. Pernthaler, S. Swidsinski, V. Loening–Baucke, M. Ortner, J. Weber, U. Hoffmann, S. Schreiber, and M. Dietel. Mucosal flora in inflammatory bowel disease. Gastroenterology 122:44-54. 2002.

21. United States Department of Agriculture. Layers 2013 Part I: Reference of Health and Management Practices on Table-Egg Farms in the United States, 2013. In. USDA-APHIS-VS-CEAH-NAHMS. 2014.

22. Vejborg, R. M., V. Hancock, A. M. Petersen, K. A. Krogfelt, and P. Klemm. Comparative genomics of Escherichia coli isolated from patients with inflammatory bowel disease. BMC genomics 12:1-12. 2011.

23. Villegas, A. M., R. Berghaus, G. Zavala, M. A. Barrios, and M. França. Profiling of egg layer flocks affected with focal duodenal necrosis based on a questionnaire survey. Avian diseases 62:241-246. 2018.

24. Villegas, A. M., L. Stabler, R. J. Moore, F. A. Uzal, J. A. Lacey, C. Hofacre, M. Lee, N. Ferguson-Noel, R. Barber, C.-S. Rimet, M. Franca. Focal duodenal necrosis in chickens: attempts to reproduce the disease experimentally and diagnostic considerations. Journal of Veterinary Diagnostic Investigation 32:268-276. 2020.

Figure 1. The percentage of top five total count for each phylum in both FDN and control samples.

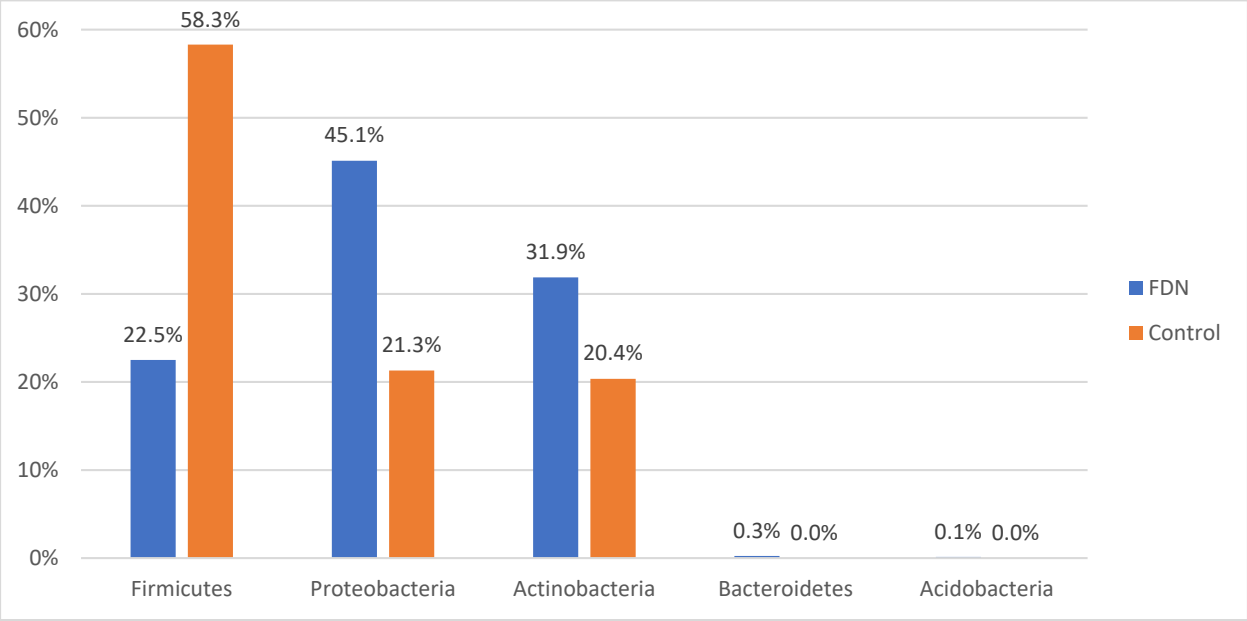
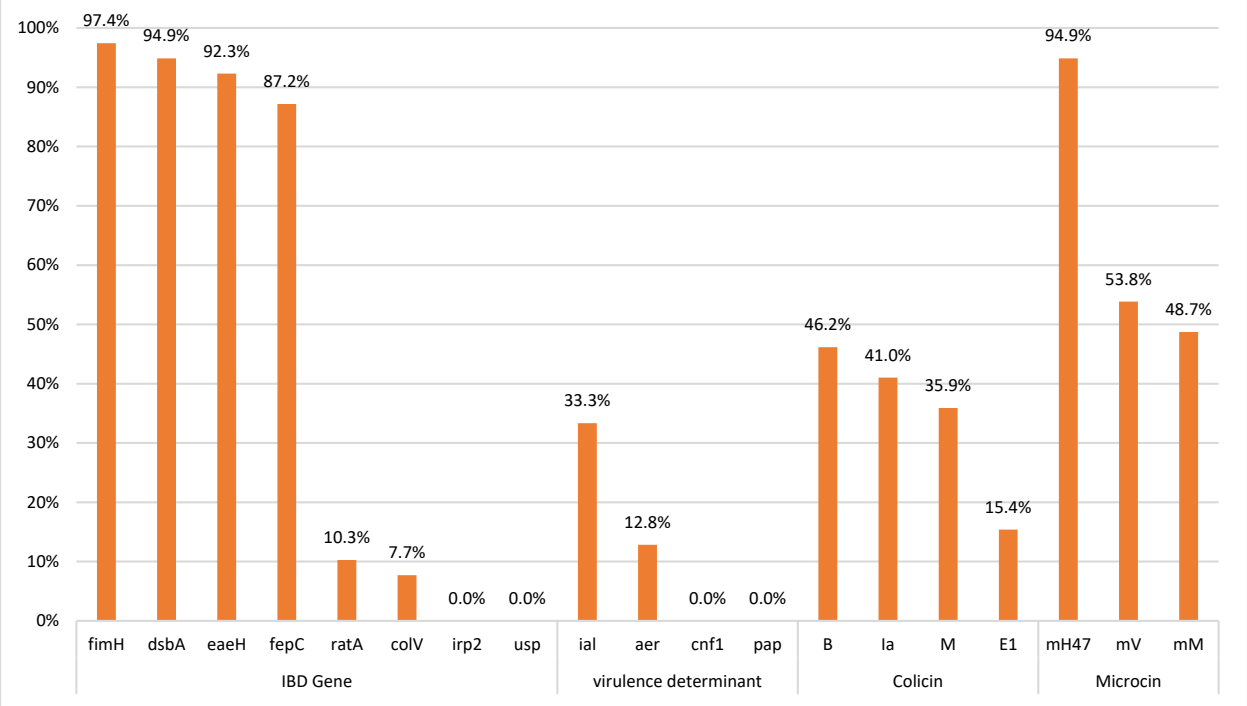


Figure 2. Inflammatory Bowel Disease (IBD) related genes PCR results.



BROILER BREEDER ADENOVIRUS TITER MONITORING DURING NATURAL CONTAMINATION OR CONTACT EXPOSURE TO A 8B STRAIN OF ADENOVIRUS

D. Venne[A]

[A]Couvoir Scott Ltée, 1798 route Kennedy, Scott (Québec), G0S 3G0, Canada

SUMMARY

Inclusion body hepatitis was controlled for many years by the use of autogenous vaccines. Recently, antibody titers have been more variable and IBH 8b outbreaks have been seen in these low antibody titer flocks. A database of serologic results was data-mined and information was used to evaluate the effect of live priming with the 8b strain on antibody titers. This study will compare antibody titers in flocks with low titers and what happens to the progeny when they seroconvert. It will also show seroconversion in inoculated birds with a semi-purified liver extract and contact birds.

MATERIALS AND METHODS

Blood and egg yolk extracts were collected and sent for analysis for antibodies agains adenovirus group 1, adenovirus 8a and adenovirus 11 with commercial ELISA kits at the diagnostic laboratory of the veterinary faculty of the Université de Montréal. Livers from clinical cases of 8b inclusion body hepatitis were semi purified by chloroform extraction and microfiltration. The extract was tested by PCR and confirmed to contain the 8b strain. They were inoculated *per os* to birds that were identified and placed amongst non inoculated birds. The inoculated and contact exposed birds were retested three weeks post inoculation and throughout the production cycle and their progeny was monitored for clinical outbreaks of inclusion body hepatitis.

RESULTS

Comparison of titers by age group showed significant effect of age on titers. Titers of birds prior to the first killed vaccine at 10 weeks of age were the lowest at 6749a. After the first killed vaccine but before the second killed vaccine they were numerically higher but highly variable and not statistically different at 14449a. They increased after the second killed vaccine at 16336b. Increased until prior to spiking 18245c decreased at the time of spiking 17856bc and were at their highest six weeks post spiking at 21913d.

For flocks prior to spiking and after spiking were 16612a and 19448b respectively.

Comparison of antibody titers for Adenovirus group1 between eggs from US suppliers that tend to grow replacement pullets on reused litter and tend not to use inactivated vaccines prior to lay. There were no significant differences in titers for us eggs vs local eggs having received two inactivated vaccines.

Exposition to semi purified liver extract between 10 and 20 weeks of age induced a seroconversion in inoculated birds and contact exposed birds. Titers prior to exposition were 9566c and increased to 19975a in inoculated birds three weeks post exposition. Contact exposed birds seroconverted to 18077a. Non-exposed birds in the same age range had titers of 13013b.

The study of this data indicates that group 1 ELISA test shows seroconversion of breeders after spiking even if they have received two killed vaccines. It can also detect type 8b passage in paired sera for inoculated and contact exposed birds. It also shows less variation in titers for birds receiving a live priming compared to that have only received inactivated vaccines. Further monitoring will show if live priming with a 8b strain induces more persistent titers than two inactivated vaccines.

IDENTIFICATION OF NOVEL AVIAN PATHOGENIC *ESCHERICHIA COLI* BIOFILM FORMATION GENES

M. Young[A], A. de Oliveira[A,B], L. Nolan[C], C. Logue[A], and N. Barbieri[A]

[A]Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602
[B]Department of Microbiology, Franklin College of Arts and Sciences, University of Georgia, Athens, GA 30602
[C]Department of Infectious Disease, College of Veterinary Medicine, University of Georgia, Athens, GA 30602

SUMMARY

Avian pathogenic *Escherichia coli* (APEC) is the etiological agent of avian colibacillosis, a leading bacterial cause of morbidity and mortality in the poultry industry worldwide. Although most *E. coli* can be found as harmless commensal organisms, APEC harbor specialized virulence factors distinguished from other *E. coli* strains that promote survival in or on the host and function in adhesion, invasion, and nutrient uptake. A key component of survival and persistence is through the formation of biofilms. Although much research has been conducted on *E. coli* biofilms, it is still unclear which genes are involved in the biofilm formation process of and are most prevalent in APEC. Therefore, the objective of this study was to identify novel genes involved in the biofilm formation ability of APEC.

A total of 15,660 mutants of a well-characterized APEC serogroup O18, ST95 strain (APEC 380) were randomly created using the signature tagged mutagenesis technique and evaluated for decreased biofilm formation ability using the crystal violet assay. Mutants with a >50% decrease in biofilm formation ability compared to the wild type were sequenced around the transposon insertion and analyzed with BLAST-N for putative biofilm formation genes. A total of 547 putative biofilm formation genes were identified. To determine which genes were most important in APEC biofilms, 30 of the identified genes were analyzed via PCR for prevalence in 109 APEC and 104 avian fecal *E. coli* (AFEC) isolates. A total of nine genes had significantly higher prevalence ($p < 0.05$) in APEC than AFEC isolates.

The selected genes are associated with several cellular processes, such as nutrient uptake and energy production, and some have unknown functions. The presence of these genes in APEC at a significantly greater rate than AFEC suggests that these genes are important in APEC biofilm formation and can be used as potential targets for antimicrobials and other therapeutics without disrupting commensal *E. coli*. Further research will evaluate the importance of these genes throughout different phases of biofilm production using planktonic growth and biofilm assays.

INTRODUCTION

Avian colibacillosis is one of the leading causes of economic loss to the poultry industry worldwide. Avian pathogenic *Escherichia coli* (APEC) is the etiological agent of avian colibacillosis that refers to localized or systemic infection resulting in syndromes such as airsacculitis, colisepticemia, peritonitis, and omphalitis, among others. There are many virulence factors associated with APEC, including the ability to persist in the environment, resist antimicrobials, and acquire genetic information from other microbes (1, 2). Biofilm is known to contribute to these virulence factors, though the exact role is still not yet well understood.

A biofilm is composed of attached or sessile microorganisms that secrete exopolysaccharides, protein, and nucleic acids to form the extracellular biofilm matrix (3). Biofilm formation can be divided into three main stages: i. attachment to a surface, ii. aggregation and formation of mature biofilm architecture, and iii. dispersion from structure (4). Biofilm formation is a response to stressful conditions and serves as a physical barrier protecting against harmful environmental factors, including antimicrobials, host defenses, and predation (5). Biofilms also provide a safe environment for horizontal gene transfer and increases conjugation efficiency between bacterial cells, which can lead to increased transfer of antimicrobial resistance and other virulence genes (6, 7).

Although much research has been done on biofilms, few genes are known to be involved in the biofilm formation process of APEC. The approach of this study can be used to uncover new genes involved in the biofilm formation process and to determine if they are most widespread and important in APEC. By discovering the genetic basis for biofilm formation in APEC, new targets can be identified for vaccines and other therapeutics.

MATERIALS AND METHODS

Strains and mutant generation. A random mutant library was generated using the signature tagged mutagenesis (STM) technique using APEC 380 as the wild-type template. APEC 380 was isolated from the pericardial and lung tissue of a chicken diagnosed with colibacillosis, and the full genome is available at CP006830 (8). Insertion mutagenesis was performed randomly using transposon pUTmini-Tn5km2 as previously described (9).

Biofilm assays for impaired biofilm formation. The mutant libraries were tested for biofilm formation abilities in M63 minimal media using the standard crystal violet assay as previously described (10). The biofilm of each mutant was measured once after a 24-hour static incubation. All tests were carried out in triplicate, and the results were averaged. Mutants with an optical density at 600 nm (OD_{600}) less than 50% of the positive control (APEC 380) were tested a second time to confirm low biofilm production. Mutants that produced less than 50% of the positive control a second time were sent for Sanger sequencing around the transposon insertion site to identify any putative biofilm formation genes.

Prevalence analysis. The prevalence of 30 selected putative biofilm formation genes was determined in a sample of 109 APEC and 104 avian fecal *E. coli* (AFEC) isolates collected from previous studies (11). Using Primer3 and Geneious Prime, six multiplex PCR primer pools of five genes each were designed to evaluate the prevalence of the selected genes.

Construction of mutants and complemented strains. Isogenic mutants of the putative APEC biofilm formation genes were constructed using λ red mutagenesis (12). Colonies were screened by PCR to identify deletion mutants and then confirmed using sequencing. Complementation was performed by cloning PCR-amplified genes of interest into the BamHI and SalI restriction sites of plasmid pACYC184. The cloned plasmids were confirmed via sequencing, then electroporated into their mutant counterparts. Complements were screened via PCR and confirmed via sequencing.

Growth curve assays. The growth curves of the APEC 380 WT, mutants, and complemented strains in M63 minimal media and Luria-Bertani (LB) broth were analyzed. Briefly, strains were incubated overnight at 37°C in LB broth. Overnight cultures were diluted to an OD_{595} of 0.05 in either M63 or LB to a total volume of 200 µL in 96-well plates. Cultures were incubated at 37°C with shaking, and OD_{595} measurements were obtained every 10 min. Growth curves were performed with four biological replicates and repeated three times on three different days. The absorbance data was averaged and plotted against time to build the growth curves. Growth curves were carried out for a total of 8 hours.

Biofilm formation assays. The biofilm formation of the mutants and complemented strains was analyzed using the standard crystal violet assay as previously described (10). Strains were incubated overnight in LB broth at 37°C. The overnight cultures were diluted 1:100 in M63 minimal media to a total volume of 200 µL in a 96-well plate and incubated for 24 hours at 37°C. Biofilm formation was read using OD_{600}. The experiment was performed with 8 biological replicates, and the absorbance data was averaged for the final result.

RESULTS AND DISCUSSION

Mutant sequence analysis. A total of 15,660 mutants were randomly generated, which is three times the number of genes in the APEC 380 wild type genome. This gave a 99% confidence that there was at least one mutant per gene in the genome. Of these mutants, 2,286 (15%) had confirmed decreased biofilm formation (<50% of the positive control) and were sent for Sanger sequencing. Of the mutants sequenced, 920 mutant sequences were analyzed using BLAST-N, resulting in a total of 547 genes of interest. From the 547 genes identified from this analysis, some genes previously described as being associated with biofilm formation were found. As both type I fimbriae and curli are important in biofilm formation (13), genes identified in this analysis included *fimA, fimC, fimD, fimF, fimG, fimH*, and *fimI* and transcriptional regulator *csgD*, which is a major biofilm regulator in both *E. coli* and *Salmonella* spp. (14). Multiple outer membrane proteins were also found, including *pgaA, pgaB, ompA*, and both *envZ* and *ompR* of the EnvZ-OmpR two-component regulatory system, all of which have been shown to be involved in or regulate biofilm formation (15 ,16, 17). Other virulence genes were also found, including hemolysin co-regulated protein (Hcp) subunit *hcp1*. Hcp is a major component of the type VI secretion system, which is a virulence factor of *E. coli* involved in adherence and pathogenicity (18). Other genes not known to be involved in biofilm formation were also identified, such as proton and nutrient transporters, hydrolases, and uncharacterized genes. Genes of interest were organized according to frequency of occurrence in the mutant sequences, and 30 genes not known to be involved in the biofilm formation process were selected for prevalence analysis.

Prevalence analysis. In this study, 213 *E. coli* (109 APEC and 104 AFEC) isolates were examined for the presence of 30 putative biofilm formation genes. PCR analysis found that nine of the tested genes

were both numerically and statistically ($p < 0.05$) significantly more prevalent in APEC than AFEC isolates (Figure 1). Statistical analysis was conducted using a two-sample t-test. The prevalence of these genes in the APEC isolates indicates that they are more widespread and likely important in APEC biofilm formation.

Growth curve assay. A preliminary growth curve was done using the APEC 380 WT, a *lacZ* deletion mutant of the WT, and the constructed isogenic mutants in LB media (Figure 2). All but two of the mutants did not differ significantly in their growth rates from the WT. The similarity in growth curves indicates that the genes of interest play roles more so in the biofilm production of APEC rather than the planktonic movement.

CONCLUSION AND FUTURE AIMS

In this study, nine new genes were found to be associated with APEC biofilm formation. Isogenic mutants and complements of these genes were constructed and will be used to evaluate biofilm formation ability and planktonic growth ability in order to elucidate the function of the identified putative biofilm formation genes.

ACKNOWLEDGMENTS

Funding for this study is provided by the University of Georgia College of Veterinary Medicine and Provost's Office.

REFERENCES

1. Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J Clin Microbiol 46: 3987–3996. 2008.

2. Tuntufye HN, Lebeer S, Gwakisa PS, Goddeeris BM. Identification of Avian pathogenic *Escherichia coli* genes that are induced in vivo during infection in chickens. Appl Environ Microbiol 78: 3343–3351. 2012.

3. Beloin C, Roux A, Ghigo JM. *Escherichia coli* biofilms. Curr Top Microbiol Immunol 322: 249–289. 2008.

4. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol 54: 49–79. 2000.

5. Kumar A, Alam A, Rani M, Ehtesham NZ, Hasnain SE. Biofilms: Survival and defense strategy for pathogens. Int J Med Microbiol 307: 481–489. 2017.

6. Lécuyer F, Bourassa JS, Gélinas M, Charron-Lamoureux V, Burrus V, Beauregard PB. Biofilm Formation Drives Transfer of the Conjugative Element ICEBs1 in *Bacillus subtilis*. mSphere 3: e00473-18. 2018.

7. Tanner WD, Atkinson RM, Goel RK, Toleman MA, Benson LS, Porucznik CA, VanDerslice JA. Horizontal transfer of the blaNDM-1 gene to *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in biofilms. FEMS Microbiol Lett 364. 2017.

8. Nicholson BA, Wannemuehler YM, Logue CM, Li G, Nolan LK. Complete Genome Sequence of the Avian-Pathogenic *Escherichia coli* Strain APEC O18. Genome Announc 4: e01213-16. 2016.

9. Li G, Laturnus C, Ewers C, Wieler LH. Identification of genes required for avian *Escherichia coli* septicemia by signature-tagged mutagenesis. Infect Immun 73: 2818–2827. 2005.

10. Skyberg JA, Siek KE, Doetkott C, Nolan LK. Biofilm formation by avian *Escherichia coli* in relation to media, source and phylogeny. J Appl Microbiol 102: 548–554. 2007.

11. Newman DM, Barbieri NL, de Oliveira AL, Willis D, Nolan LK, Logue CM. Characterizing avian pathogenic *Escherichia coli* (APEC) from colibacillosis cases, 2018. PeerJ 9: e11025. 2021.

12. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645. 2000.

13. Klemm P, Schembri M. Type 1 Fimbriae, Curli, and Antigen 43: Adhesion, Colonization, and Biofilm Formation. EcoSal Plus 1. 2004.

14. Römling U, Bian Z, Hammar M, Sierralta WD, Normark S. Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. J Bacteriol 180: 722–731. 1998.

15. Itoh Y, Rice JD, Goller C, Pannuri A, Taylor J, Meisner J, Beveridge TJ, Preston JF, Romeo T. Roles of *pgaABCD* genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-beta-1,6-N-acetyl-D-glucosamine. J Bacteriol 190: 3670–3680. 2008.

16. Ma Q, Wood TK. OmpA influences *Escherichia coli* biofilm formation by repressing cellulose production through the CpxRA two-component system. Environ Microbiol 11: 2735–2746. 2009.

17. Vidal O, Longin R, Prigent-Combaret C, Dorel C, Hooreman M, Lejeune P. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. J Bacteriol 180: 2442–2449. 1998.

18. Navarro-Garcia F, Ruiz-Perez F, Cataldi A, and Larzábal M. Type VI Secretion System in Pathogenic *Escherichia coli*: Structure, Role in Virulence, and Acquisition. Front Microbiol 10: 1965. 2019.

Figure 1. Prevalence analysis results of selected putative biofilm formation genes in APEC and AFEC isolates. The asterisk (*) indicates a significance of $p < 0.05$.

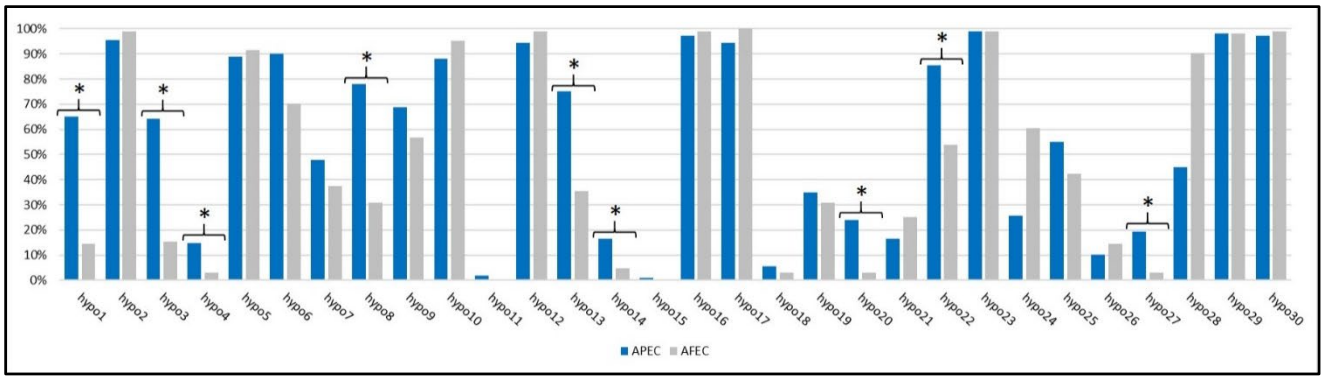


Figure 2. Preliminary growth curve of the APEC 380 WT (red), APEC 380 ΔlacZ (blue), and the isogenic mutants of the genes of interest (gray).