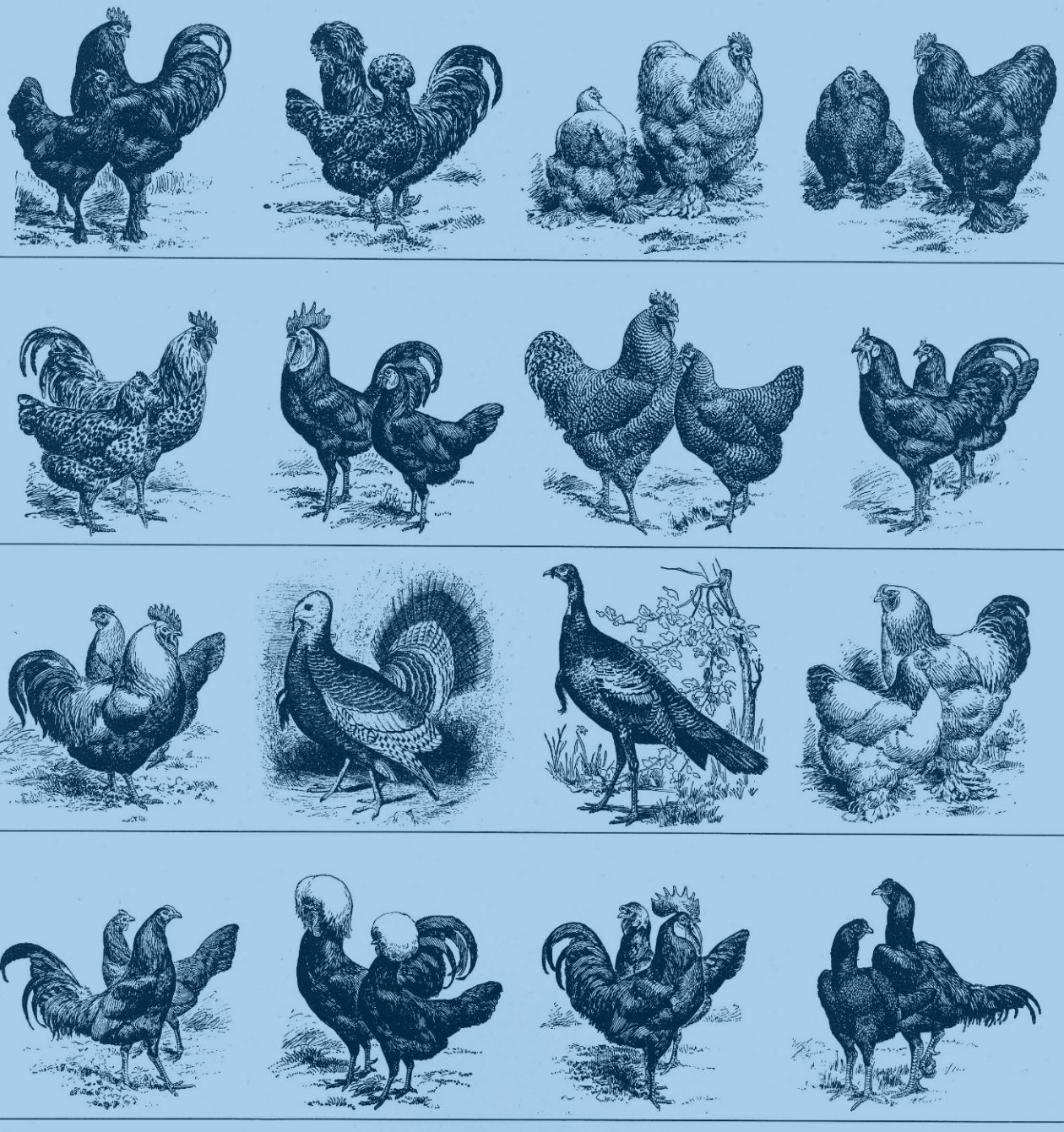
PROCEEDINGS OF THE SIXTY-NINTH WESTERN POULTRY DISEASE CONFERENCE

March 30 – April 1, 2020 Sacramento, CA

(Published proceedings only; the actual conference was not held because of a concurrent coronavirus pandemic.)

WESTERN POULTRY
DISEASE CONFERENCE



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UNIVERSITY OF CALIFORNIA, DAVIS

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April 7, 2020

Dear Colleagues,

The global COVID-19 pandemic has had an unprecedented, far-reaching impact to individuals and organizations worldwide. As a result, many conferences, including the WPDC canceled annual scientific meetings in 2020. The WPDC executive committee would like to thank all researchers and coauthors that submitted abstracts for the 69th annual meeting. We acknowledge the hard work our researchers have put into preparation of informative manuscripts and have elected to publish all manuscripts in the 69th annual WPDC proceedings. Despite the cancellation of the 2020 meeting, we remain committed to facilitating the dissemination of novel and informative poultry research and case reports with the publication of the submitted manuscripts. The WPDC executive committee would also like to acknowledge our sponsors that have made contributions and pledged support for future meetings.

69th WPDC Executive Committee

69th WPDC SPECIAL RECOGNITION AWARD

MARK C. BLAND

Dr. Mark Bland grew up on a small farm in a rural area near Hillsboro, Oregon. He showed an early propensity for kinship with the feathered fauna by raising a small flock of laying hens with his grandmother. This later escalated into caring for a flock of 500 mixed breed layers as an FFA project on his grandmother's farm.

In his early collegiate days, Mark was deciding what to do with his life. One day he happened to pass the poultry science building (Dryden Hall) on Oregon State University's campus. He was intrigued, so he entered the doors of the venerable Hall and was met by a kind Dr. George Arscott, Poultry Science Department Head. Dr. Arscott took Mark under his wing and helped provide him with scholarship opportunities so he could afford his studies. Mark knew from that moment that poultry science and medicine was what he wanted to do as a career. He is a loyal Beaver, having completed both his BS and MS degrees in poultry science at Oregon State.

After graduating from veterinary school at Oregon State University in 1987, Mark completed a residency in poultry medicine at the University of California, Davis. Mark worked as a corporate veterinarian for Nicholas Turkey Breeding Farms, Inc. from 1988 to 1997. After leaving NTBF, he became a poultry veterinary consultant with Cutler Associates International, which gave him the opportunity to expand his knowledge and expertise in many facets of the poultry industry. He also provided part-time poultry diagnostic services at the California Animal Health & Food Safety Laboratory in Turlock, CA, from 1998 to 2008. Additionally, he is involved with pesticide and chemical training programs, developing meat and egg quality assurance plans, FDA and CDFA eggshell safety programs, avian influenza response plans, and other various county and state

regulatory and environmental issues. His extensive work in the US and Canada has made Mark one of the most knowledgeable and sought-after poultry veterinarians in North America.

Dr. Bland is board certified in the American College of Poultry Veterinarians. He has served in many leadership positions, including president of the Association of Turkey Veterinarians (1995) and president of the AAAP (2013). He was the recipient of the Pacific Egg and Poultry Association Poultry Scientist of the Year Award (2006) and the California Poultry Industry Federation's Pioneer Award (2019).

Over the years, Dr. Bland has been an active supporter of the WPDC, having served a memorable year as Program Chair of the combined 45th WPDC/XXI ANECA meeting in Cancun, Mexico (1996). He followed up as WPDC President the following year (1997).

Although Dr. Bland is known for being an accomplished veterinarian, his most notable trait is his willingness to teach and help others. Mark dedicates many unpaid hours every year training residents, interns, and veterinary students who have an interest in poultry. This commitment to students is reflected in his on-going efforts to increase scholarship availability for students to travel and have externship experiences. Mark assists in providing externship opportunities for ten to fourteen students each year to come and travel to farms with him. He even welcomes students to stay at his home in Napa when they are not traveling. For the CAHFS Turlock residency program, he does multiple field trips to poultry farms with residents and dedicates about 30 hours of individual teaching time to prepare them for ACPV boards. This is all unpaid teaching. Mark gives yearly guest lectures at Oregon State University Veterinary School and invites the Poultry Science undergraduates to the lectures. He also lectures at the School of Veterinary Medicine at UC Davis and occasionally at the Washington State University Veterinary School. Dr. Bland's class in poultry medicine is sometimes the only poultry instruction these veterinary students receive.

He and his lovely wife of 36 years, Karen, live in Napa, CA and have two children (Dori and Michael) and one grandson (Bode). Michael and his wife, Alexis, live in Oakland, CA; Dori, her husband Josh, and son Bode reside near Boulder, CO.

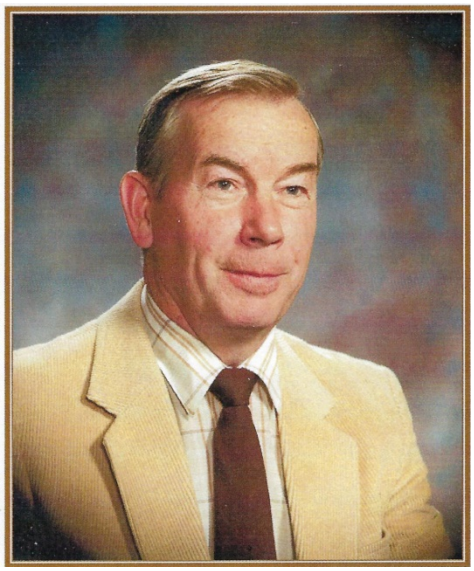
Mark and Karen love to travel and have been to many memorable places, such as New Zealand, Africa, Patagonia, Indonesia, Alaska, and Montana. They hope to visit many more places in the years to come.

It could be said that two of Mark's greatest passions are fishing and milkshakes, but not necessarily in that order. He loves to share a milkshake with two-year-old Bode and can't wait to start taking him fishing. One of Mark's favorite lures is the "Blue Bomber" (at least when fishing at Pete's Hole in Utah).

It is a pleasure and privilege to present Dr. Mark C. Bland with the 69th Western Poultry Disease Conference Special Recognition Award.

IN MEMORIAM

MARCUS M. JENSEN



Marcus Martin Jensen, 90, of Provo, Utah, passed away at home on November 23, 2019. He was born May 26, 1929, in Mantua, Utah. His early life was spent learning the value of hard work living on a small farm during the depression years. Marcus was a faithful member of the Church of Jesus Christ of Latter-Day Saints, serving in many different callings and assignments within the Church.

Marcus enlisted in the U.S. Army in 1947 and upon his return attended Utah State University, graduating with a Master's Degree in Microbiology in 1953. He subsequently completed a Ph.D. program in medical microbiology at the University of California, Los Angeles Medical School in 1961. He stayed on as a research associate at UCLA until accepting a position in the Department of Microbiology at Brigham Young University in 1969.

His research at BYU soon led him back to his agrarian roots, which created opportunities of collaborative interaction with the Utah turkey industry. Over the years, he developed three successful vaccines for the poultry industry that were widely used internationally. These vaccines were some of the first products developed at BYU to bring in royalties to the University and thereby helped to establish BYU's Technology Transfer Office.

Dr. Jensen had his first introduction to the Western Poultry Disease Conference in 1978, when he substituted for Dr. Royal Bagley in presenting their findings on a possible cause of the elusive "coryza" problem then facing the Utah turkey industry. Marcus commented, "So, I attended my first WPDC, and there met Dr. Arnold 'Rosy' Rosenwald and other workers in the field of avian diseases. Little did I realize at that time that I would spend the remainder of my career working closely with these persons." He remained active in the WPDC until his retirement in 2006, having served as Program Chair and President (1987 and 1988, respectively).

In 1986, Dr. Jensen took on the additional role of Proceedings Editor for the WPDC. With the help of Scholarly Publications and the Graphics Communications Departments at BYU, participating authors' manuscripts were collected using computer disks or received directly over the BITNET (an early internet system connecting most universities in the world at the time). This allowed the printed proceedings to be available for the first time on-site at the 35th WPDC/ANECA meeting in Puerto Vallarta, Mexico. He continued to serve as Proceedings Editor providing the published proceedings at the time of the conference through the 1996 combined WPDC/ANECA meeting in Cancun, Mexico. The proceedings were mailed to the meeting venue on years the WPDC was not located in California. For the California meetings, Dr. Jensen would load up a university van full of proceedings, graduate students, and a local turkey veterinarian and make the often hair-raising trek across the Sierra Nevada. Much of the construction design and legacy associated with the current WPDC Proceedings can be attributed to Marcus' creative foresight.

Dr. Jensen's greatest attributes were his genuine concern and exceptional kindness toward his graduate students and colleagues. When asked what were some of the big challenges in his career, Marcus commented that perhaps the most challenging thing is to get the industry to readily embrace available technology. When asked what message would he give to the next generation, his simple advice was to "keep at it." Throughout his career at BYU, Marcus was actively involved in national and international poultry disease organizations, which took him to many different countries. A supportive family and successful career allowed Marcus and Mary Jensen to do what they enjoyed most: travel the world!

We wish to give a special heart-felt thanks to these sponsors listed on the next few pages that have contributed so generously and given their 2020 pledge to the 70th Western Poultry Disease Conference, and for those who give their continuous support over the years!

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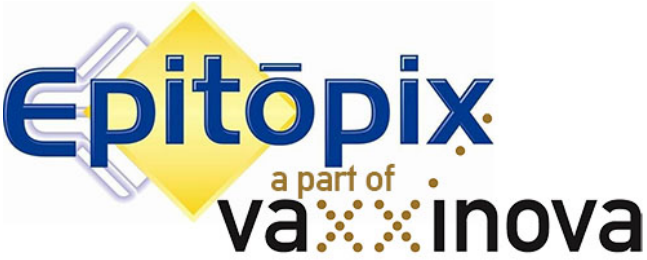
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Rodrigo Gallardo
Poultry Medicine Laboratory at UCD

SPECIAL ACKNOWLEDGEMENTS

The 69th 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 to help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. 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. All our contributors and supporters are listed on the previous pages. We greatly appreciate their generosity and sincerely thank them and their representatives for supporting this year's meeting of WPDC.

Dr. Simone Stoute, Program Chair of the 69th WPDC, would like to thank the WPDC Executive Committee members and Shelly Popowich for their support and assistance in organizing this meeting. She also wishes to thank all student volunteers and moderators for their willingness to provide assistance during the conference. On behalf of the WDPC, Dr. Stoute also extends her sincere gratitude to all invited speakers who have contributed to this year's conference.

Many have provided special services that contribute to the continued success of this conference. For this year's meeting, the WPDC has contracted Conference and Events Services, of the University of California, Davis, for providing budgetary and registration support for the conference. We would like to thank Ms. Teresa Alameda, Ms. Lina Layiktez, and the staff at Conference & Event Services for their exceptional work with our conference. Again, we thank Bob and Janece Bevans-Kerr and AAAP for their continual support.

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.

We unanimously acclaim the extraordinary efforts of Dr. Rodrigo Gallardo and UCD for assisting with the cancellation of the meeting and the cancellation of the hotel contract. We are indeed grateful for the extra time and work that were expended in resolving these difficult issues in a timely manner.

We wish to express a very special thanks to the ACPV for taking care of our proceedings on the web site, helping to provide administrative duties, and to provide CE for attendance at the WPDC!

69th WESTERN POULTRY DISEASE CONFERENCE OFFICERS

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69th WPDC PROCEEDINGS

Please note that the proceedings of the 69th 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 69th 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	J. W. Dunsing	G. Yan Ghazikhanian	P. P. Levine	
5th ANECA	Angel Mosqueda T.			
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 39th ANECA	P. Cortez Néstor Ledezma M.	Ernesto Soto Ernesto Soto	Hugo Medina 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 Chin
67th WPDC – 2018	C.G. Sentíes-Cué	Rodrigo A. Gallardo		David D. Frame
68th WPDC – 2019 44th ANECA	R. Gallardo Ricardo Cuetos Collado	Sarah Mize Maritza Tamayo		Gregg Cutler
69th WPDC – 2020	S. Mize	Simone T. Stoute		Mark C. Bland

MINUTES OF THE 68TH WPDC ANNUAL BUSINESS MEETING

President Rodrigo Gallardo called the meeting to order on Friday April 5, 2019 at 12:30 PM, at the Sheraton Buganvilias Resort and Convention Center, Puerto Vallarta, Mexico. There were approximately 15 people in attendance.

APPROVAL OF THE 67TH WPDC BUSINESS MEETING MINUTES

The minutes of the 67th WPDC business meeting were reviewed by members of the Executive Committee during the executive committee meeting on Tuesday April 2, 2019, and were recommended for approval as written. Dr. Gregg Cutler motioned that the minutes of the 67th WPDC business meeting be approved as presented. Dr. Nancy Reimers seconded. Motion passed.

ANNOUNCEMENTS

President Gallardo acknowledged all 20 contributors at the Benefactor, Patron, Donor, and Sustaining Members levels. All were acknowledged and thanked for their support. Dr. Nancy Reimers was suggested as the Contribution Chair, she accepted the position.

REPORT OF THE SECRETARY-TREASURER

The Secretary-Treasurer report was presented by Dr. Rodrigo Gallardo.

- 66th WPDC in 2017 had 41 contributors and $31,000
- 67th WPDC in 2018 had 37 contributors and $34,950
- 68th WPDC there were 20 contributors and $26,050

Additionally, it was noted that CEVA did not contribute this year. The CEVS account currently has a balance of $87,586 (including contributions until April 1, 2019) and the PHR UCD account has a balance of $44,030. The current balance of both accounts is $131,616 in comparison to $107,000 in 2018.

REPORT OF THE PROCEEDINGS EDITOR

The Proceedings Editors' report was presented by Dr. Rodrigo Gallardo on behalf of Dr. David Frame. There were 182 pages for publication in the proceedings. Issues with papers submitted late and presentations not loaded prior to the session were announced, however these issues will be addressed for the future.

WPDC is grateful to the American College of Poultry Veterinarians (ACPV) for providing the Proceedings on their website at acpv.info, which are open to the public.

FUTURE MEETINGS

Future WPDC meeting dates and locations were discussed:

2020: 69th WPDC and ACPV-sponsored workshop, Sacramento, CA March 29 to April 1, 2020
2021: 70th WPDC and ACPV-sponsored workshop, Sacramento, CA March 14 to March 17, 2021
2022: 71st WPDC and ACPV-sponsored workshop, Vancouver, BC April 9 to April 13, 2022

Dr. Nancy Reimers motioned that the 71st WPDC and ACPV-sponsored workshop be held in Vancouver, BC, Dr. HL Shivaprasad seconded. Motion passed.

WPDC EXECUTIVE COMMITTEE

Current WPDC officers were acknowledged for their work and participation. A plaque was presented to the exiting President. Dr. Simone Stoute was named as the Program Chair for the 69th WPDC.

Nomination and election of Program Chair of 70th WPDC: Dr. Lynn Bagley was nominated as the 70th WPDC Program Chair by Dr. Rodrigo Gallardo on behalf of Dr. David Frame. Dr. Sarah Mize made the affirmative motion, which was seconded by Dr. HL Shivaprasad. Motion passed.

The following officers were presented for 2020:

Program Chair: Dr. Simone Stoute
President: Dr. Sarah Mize
Past-President: Dr. Rodrigo Gallardo
Contributions Chair: Dr. Nancy Reimers
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Rodrigo Gallardo
Program Chair-elect: Dr. Lynn Bagley

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

NEW BUSINESS

Dr. Gallardo stated that continuing education credits will be provided by ACPV. Each attendee will receive an email with a link to download.

Dr. Gregg Cutler was awarded the 68th WPDC Special Recognition Award by Dr. Rodrigo Gallardo.

Possible student scholarships were discussed that would allow one to two students (DVM, graduate or resident) to attend WPDC with travel support, free registration etc., selection based on call for titles. These scholarships would be funded through the WPDC account or through contributors' support.

The new WPDC logo is to be used for future meetings and correspondence.

Registration will now all be online because of the higher costs of mailing and out-of-date registration lists. Registrants from the previous four to five years will be contacted.

A Meeting Support Chair to assist Dr. Rodrigo Gallardo in his position as Secretary-Treasurer was discussed. Dr. Reimers, in her position as Contribution Chair, constitutes part of this additional needed support.

Dr. Rodrigo Gallardo turned the presidency over to Dr. Sarah Mize who thanked him for all of his work and support and welcomed him as the new Secretary-Treasurer. Dr. Mize adjourned the meeting at 1:00 PM.

TABLE OF CONTENTS

DYNAMICS OF INFECTIOUS BRONCHITIS VIRUSES IN COMMERCIAL BROILERS RAISED IN A HIGH DMV1639-CHALLENGE AREA

M. Alfonso[A], H.J. Roh[A], and S. Gustin[B]

[A] Ceva Animal Health, LLC. 8735 Rosehill Rd, Ste 300, Lenexa, KS 66215
[B] Tyson Foods, 2200 Don Tyson Parkway, Springdale, AR 72762

SUMMARY

The DMV1639 strain of infectious bronchitis virus (IBV) was introduced to Northwest Arkansas in winter 2017-spring 2018 and caused severe respiratory disease and airsacculitis in broilers. A broiler farm, located in a high DMV169 challenge area, with a history of increased condemnation at processing due to airsacculitis, was monitored for IBV during one-year period. General surveillance was conducted using various samples (water, serum, and tissues) to identify the underlying issues for chronic airsacculitis and confirmed the circulation of DMV 1639 as well as increased IBV titer. Several changes in the Infectious Bronchitis vaccination program were introduced during this time in order to reduce DMV1639 circulation and improve flock health.

For IBV surveillance, a total of 30 birds per flock (five birds per house) were bled, and tracheas, kidneys, and cecal tonsils were collected separately at processing age (42-45 days) in six consecutive flocks raised at the farm. IBV serology (Biocheck ELISA), as well as virus detection and quantification by qRT-PCR were performed to identify circulating IBV types. Additionally, air sac condemnation percentage and performance information was collected.

During this one-year period, changes of circulating IBV related to IBV vaccination change were observed. Air sac condemnation rate at processing plant has decreased. By the end of the monitoring period, IB ELISA titers have decreased and detection of DMV1639 from processing aged birds has significantly decreased to the point of being undetectable.

INTRODUCTION

The DMV1639 strain of infectious bronchitis virus (IBV) was first identified in commercial broiler chicken flocks in Delmarva in 2011 (DMV/1639/11). This initial isolate was nephropathogenic in susceptible chickens and it most likely originated from a 1997-200 nephropathogenic infectious bronchitis outbreak in Pennsylvania (1). Since then, viruses highly related to the DMV1639 strain have been detected in Canada (2) and other regions in the United States of America (Personal communication with Dr. Jose Linares and Dr. Sherryl Davidson). The clinical signs caused by the more recent DMV1639 isolates have shifted from nephropathogenic to respiratory disease (3,4; Personal communication with Dr. Don Ritter). An increased incidence of airsacculitis in commercial broilers was reported in North West Arkansas (NWAR) in winter 2017-spring 2018, and DVM1639 viruses were detected in tracheas, kidneys, and cecal tonsils of sick broilers and sentinel birds in this area.

A broiler farm, located in a high DMV169 challenge area in NWAR, with a history of increased condemnations at processing due to airsacculitis, was monitored for IBV during one-year period. Dynamics of the different IBV found at this farm are described in this manuscript.

MATERIALS AND METHODS

A six-house commercial broiler farm located in NWAR rearing 238,000 birds per flock started experiencing increased airsacculitis in flocks processed in January and March 2018. Birds were processed at 48 days of age. In an effort to prevent airsacculitis, a comprehensive program including water sanitation and changes to the vaccination program were implemented in the following flocks (Table 1). Water, serum, and tissue samples were collected to identify the problem. A total of 30 birds per flock (5 birds per house), were bled, and tracheas, kidneys, and cecal tonsils were collected separately at 29 and 45 days of age in a flock placed in July 2018 (Flock #1). Water samples taken from the end of the drinker lines at each house were also analyzed for bacterial contamination. Once the problem was identified, and for IBV surveillance purposes, a total of 30 birds per flock (5 birds per house) were bled, and tracheas, kidneys, and cecal tonsils were collected separately close to processing age (42-45 days) in the following five flocks raised at the farm (Flock #2-#6). IBV serology (Biocheck ELISA), as well as virus

detection and quantification by qRT-PCR were performed to identify circulating IBV types. Universal 5UTR IBV primers/probe (5), Ark, Conn, Mass, DE072-GA98 specific primers/probes (6), classical GA08, GA13 (UGA, Dr. Brian Jordan, unpublished), variant GA08 (Ceva SSIU, unpublished) and DMV primers/probe (U of Delaware DMV assay by Dr. Gelb and Dr. Ladman) were used. Additionally, airsacculitis condemnation % and performance information was collected for each flock.

RESULTS

In the first monitored flock (Flock 1), presence of IBV DMV1639 in tracheas and cecal tonsils of birds at 45 days of age was demonstrated by qRT-PCR. Mass and Ark strains, consistent with the vaccination program received by those birds, were also detected at 29 days. Ark persisted in cecal tonsils by 45 days. IBV ELISA titers increased from 29 to 45 days indicating exposure to IBV (GMT 751 vs. 3,358 at 29 and 45 days respectively). There was no serological evidence or positive detection of other respiratory pathogens (ILT, NDV, MG/MS, ORT). The existing water sanitation program was effectively controlling bacteriological contamination.

The results from Flock 1 monitoring suggested IBV as a potential source of the airsacculitis issue in the farm. Additional IBV surveillance was performed for subsequent flocks (Flocks 2-6). IBV ELISA titers and qRT-PCR IBV results are shown in Table 2 and Chart 1. In Flock 2, IBV ELISA titers peaked with GMT 13,998, and strong presence of DMV1639 detected by qRT-PCR in all samples types (kidney being the highest) confirmed the continuation of the IBV challenge in the farm. For following flocks (Flocks 3-6), GA type vaccine (Cevac IBron) replaced GA98 vaccine in hatchery vaccination program with Mass and Ark, and field boost vaccination was removed. Decreased IBV ELISA titers and a lower level of DMV1639 was detected by qRT-PCR in those flocks (Flocks 3-5) until it was not detectable in Flock 6 after continuous GA type vaccination. Variant GA08 qRT-PCR detected GA08 type vaccine (Cevac IBron) consistently in cecal tonsils since vaccination started. Mass was sporadically found in tracheas in Flocks 3, 5, and 6. Ark was predominantly found in cecal tonsils except in Flock 6.

The average airsacculitis percentage in five flocks processed between January 2018 and November 2018 was 0.056%. With the implementation of GA type vaccine at this farm, average airsacculitis in the following five flocks processed between January 2019 and October 2019 was reduced to 0.016%. When looking at the most challenging time of the year for respiratory disease, the difference was more dramatic. There was a 4.4-fold decrease in airsacculitis condemnations when comparing flocks raised at this farm during winter 2017-2018 and winter 2018-2019 (0.11% before GA type vaccination vs. 0.025% after GA type vaccination).

DISCUSSION

During this one-year period, changes of circulating IBV related to IBV vaccination change were observed. The impact of better ventilation during warmer weather might have also played a role in the viral challenge experience by the flocks. Airsacculitis condemnation rate at the processing plant has decreased. By the end of the monitoring period, and after continuous GA type vaccination, IBV ELISA titers have decreased and detection of DMV1639 from processing aged birds has significantly decreased to the point of being undetectable.

ACKNOWLEDGMENTS

Mr. Keith Childress, Mr. Kenny Russo, and Tyson Foods Noel Broiler Team for facilitating sample collection in the field. Ceva SSIU Team for all laboratory analysis and reporting.

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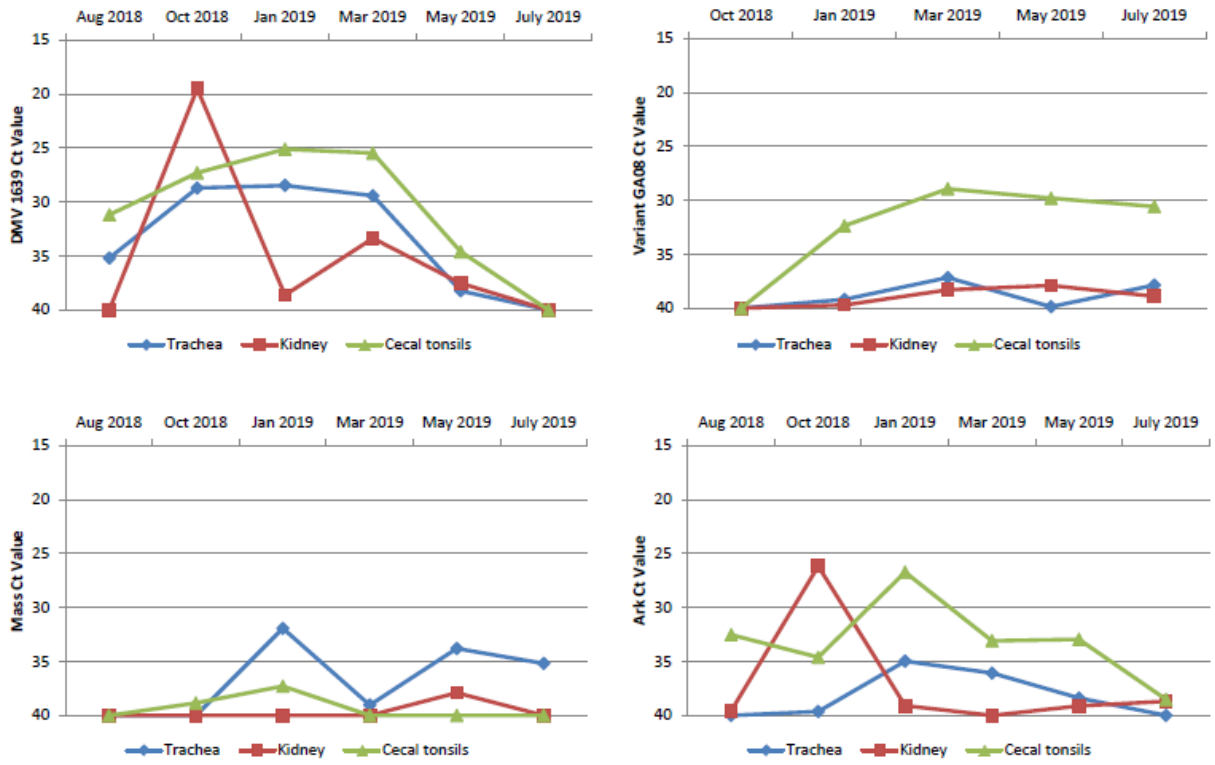
Table 1. IBV Vaccination program by flock.

Flock #	Processing Date	IBV Vaccination Program
1	8/25/2018	Day 1: Mass (1/2 dose), Ark (1/2 dose), GA98 (Full dose) Day 14: Ark (1/2 dose), GA98 (1/2 dose)
2	11/3/2018	Day 1: Mass (1/2 dose), Ark (1/2 dose), GA98 (Full dose) Day 14: Ark (1/2 dose), GA98 (1/2 dose)
3	1/12/2019	Day 1: Mass (1/2 dose), Ark (1/2 dose), GA type (Full dose)
4	3/16/2019	Day 1: Mass (1/2 dose), Ark (1/2 dose), GA type (Full dose)
5	5/25/2019	Day 1: Mass (1/2 dose), Ark (1/2 dose), GA type (Full dose)
6	8/3/2019	Day 1: Mass (1/2 dose), Ark (1/2 dose), GA type (1/2 dose)

Table 2. IBV ELISA titers at 42-45 days of age in 6 consecutive flocks.

IBV ELISA GMT (CV)					
August 2018	October 2018	January 2019	March 2019	May 2019	July 2019
3,358 (65)	13,998 (29)	2,702 (52)	3,696 (55)	2,198 (82)	1,925 (70)

Chart 1. qRT-PCR IBV flock average Ct Value of 6 consecutive broiler flocks (Note: August and October flocks were not vaccinated with GA type).



CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES IN TANZANIA AND GHANA USING THIRD-GENERATION SEQUENCING

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SUMMARY

Virulent Newcastle disease virus (vNDV) is present worldwide, causes economically significant respiratory disease in birds, and can lead to costly outbreaks when introduced to NDV-free countries. NDV varies widely in the severity of disease it produces due to its high genetic diversity, and its pathogenicity is determined by the fusion (F) protein. In this study, we characterized Newcastle Disease Virus (NDV) isolates from various locations in Tanzania and Ghana using Oxford Nanopore MinION sequencing technology. Reverse transcription PCR was performed to amplify regions containing the cleavage site and hypervariable regions of the fusion gene, specifically a short region comprising 788 nucleotides (173 nucleotides of the 3' region of the matrix gene and 615 nucleotides of the 5' region of the fusion gene) and an overlapping long region of 1285 nucleotides (3/4 of the coding sequence of the fusion gene). The portable MinION was used to sequence the amplicons, and phylogenetic analysis indicated that the sequences belonged to genotypes V, VII, and XIII. Here, we demonstrate the utility of the MinION to rapidly sequence NDV isolates from Tanzania and Ghana.

INTRODUCTION

NDV causes economically significant disease and is endemic to many countries, where it infects at least 236 species of wild birds and poultry (1). Virulent NDV strains are classified as avian paramyxovirus 1 (APMV-1), from the family Paramyxoviridae and species avian orthoavulavirus 1 (2). The virus is shed in the feces and saliva, and infection may involve the respiratory, gastrointestinal, nervous, and reproductive systems, resulting in up to 100% mortality in susceptible chickens (1).

NDV strains are grouped into two main classes: class I and class II (3). Class I viruses are of low diversity and virulence and are mostly found in wild birds, whereas class II viruses include multiple genotypes and sub-genotypes of both low and high virulence and infect both wild birds and poultry (4, 5).

APMV-1 is a negative sense, single-stranded RNA virus with a genome size of about 15.2 kb (3) and consists of six structural proteins, listed from 3' to 5': nucleocapsid protein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (6). Genetic characterization of NDV is important for effective control of ND, and sequencing of the fusion cleavage site is commonly used to demonstrate virulence. Virulent strains have a multibasic amino acid sequence motif at the C-terminus of the F2 protein and a phenylalanine at the N-terminus of the F1 protein, which allows them to be cleaved by furin-like proteases found in tissues throughout the host (7, 8, 9).

ND outbreaks are particularly common in African countries, and there is a need for continued genetic characterization of the NDV strains circulating in various regions of Africa. Recently, an updated classification system has identified isolates from different countries in Africa as belonging to genotypes I, VI, VII, XIII, XIV, XVII, XVIII, XXI (4). In Tanzania, chickens in live bird markets from six different regions were surveyed for NDV in 2012, and the isolates were recently classified as genotype V and XIII (10). In West and Central Africa, genotypes XIV, XVII, and XVIII were detected in poultry (11), and genotypes I, VI and XVIII were identified in wild birds (12).

The aim of this study was to provide more information about NDV circulating in poultry in Tanzania and Ghana. Using MinION sequencing technology, we have rapidly sequenced the partial F gene and determined the genotypes of these new isolates.

MATERIALS AND METHODS

Clinical samples. The isolates were obtained from chickens from various locations in Tanzania and Ghana in 2018-2019 for this study. Samples were stored at -80°C.

RNA extraction and reverse transcription polymerase chain reaction. Viral RNA extraction from 50 µl of sample was performed using the MagMAX™-96 AI/ND Viral RNA Isolation Kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. The reverse transcription polymerase chain reaction (RT-PCR) was performed using the Qiagen OneStep RT-PCR Kit (Qiagen, Germantown, MD, USA), following the manufacturer's protocol. Each 50 µL reaction contained 25 µL DEPC water, 10 µL 5x buffer, 2 µL dNTP mix, 2 µL RT-PCR enzyme mix, 3 µL each of forward and reverse primers, and 5 µL of viral RNA.

Two sets of NDV-specific primers were used for virus identification. The first primer pair spanned 788 nt and has been previously published (4331F and 5090R) (13, 14). The second set of primers flanked a larger region of 1285 nt that included 75% of the coding sequence of the fusion gene and has also been previously published (15).

The RT-PCR reactions were run on a thermocycler under the following conditions: one cycle of 30 min of reverse transcription at 50°C, followed by 15 min at 95°C, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 53°C, and 2 min of extension at 72°C.

MinION library preparation. For MinION-compatible library preparation, 1 µg of each DNA amplicon was diluted in 49 µL of nuclease-free water for use with the 1D Native barcoding genomic DNA protocol (Oxford Nanopore Technologies, Oxford, UK), which was developed for the Native Barcoding Expansion sets (EXP-NBD104 and EXP-NBD114) and Ligation Sequencing Kit (SQK-LSK109). Briefly, DNA was repaired and end-prepped using the NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (New England BioLabs, Ipswich, MA, USA). End-prepped DNA was purified by AMPure XP beads (Beckman Coulter, USA), and 500 ng of each sample was diluted to a total of 22.5 µL in nuclease-free water for barcoding. Barcoded DNA was bead purified and pooled in equimolar amounts for a target pooled sample of 700 ng diluted to a total of 65 µL in nuclease-free water. Finally, pooled DNA was ligated to adapter sequences and bead purified before sequencing.

MinION sequencing. The libraries were sequenced with the MinION Nanopore sequencer. A new FLO-MIN106 R9.4 flow cell (Oxford Nanopore Technologies, Oxford, UK), stored at 4°C prior to use, was equilibrated to room temperature and primed for sequencing using the Flow Cell Priming Kit (Oxford Nanopore Technologies, Oxford, UK), following the manufacturer's instructions. The pooled DNA libraries were prepared by adding 37.5 µL Sequencing Buffer, 25.5 µL Loading Beads, and 12 µL library. The library was then added to the flow cell via the SpotON sample port. The sequencing was run for a minimum of six hours using the MinKNOW software v3.1.19. Raw reads that passed the quality filter (Q>7) were used in further data analysis.

Phylogenetic analysis. Phylogenetic analyses were performed on Geneious Prime (Geneious, USA) using the partial fusion gene assembled consensus sequences (24 sequences from each MinION run) and coding sequences provided in the Class II "pilot" tree described previously (3). Sequences were analyzed using Multiple Alignment with Fast Fourier Transformation (MAFFT v7.450). The final tree was constructed using Randomized Axelerated Maximum Likelihood (RAxML v8) based on the General Time Reversible (GTR) model, with 500 bootstrap replicates.

RESULTS

Of the 24 NDV isolates from Tanzania, all were successfully sequenced and corresponded to genotypes V, VII, and XIII (Fig. 1). Eight isolates belonged to genotype V, one isolate was classified as genotype VII, and 15 isolates were characterized as genotype XIII. Sequencing and phylogenetic results of NDV isolates from Tanzania and Ghana will be discussed in more detail at the Western Poultry Disease Conference in 2020.

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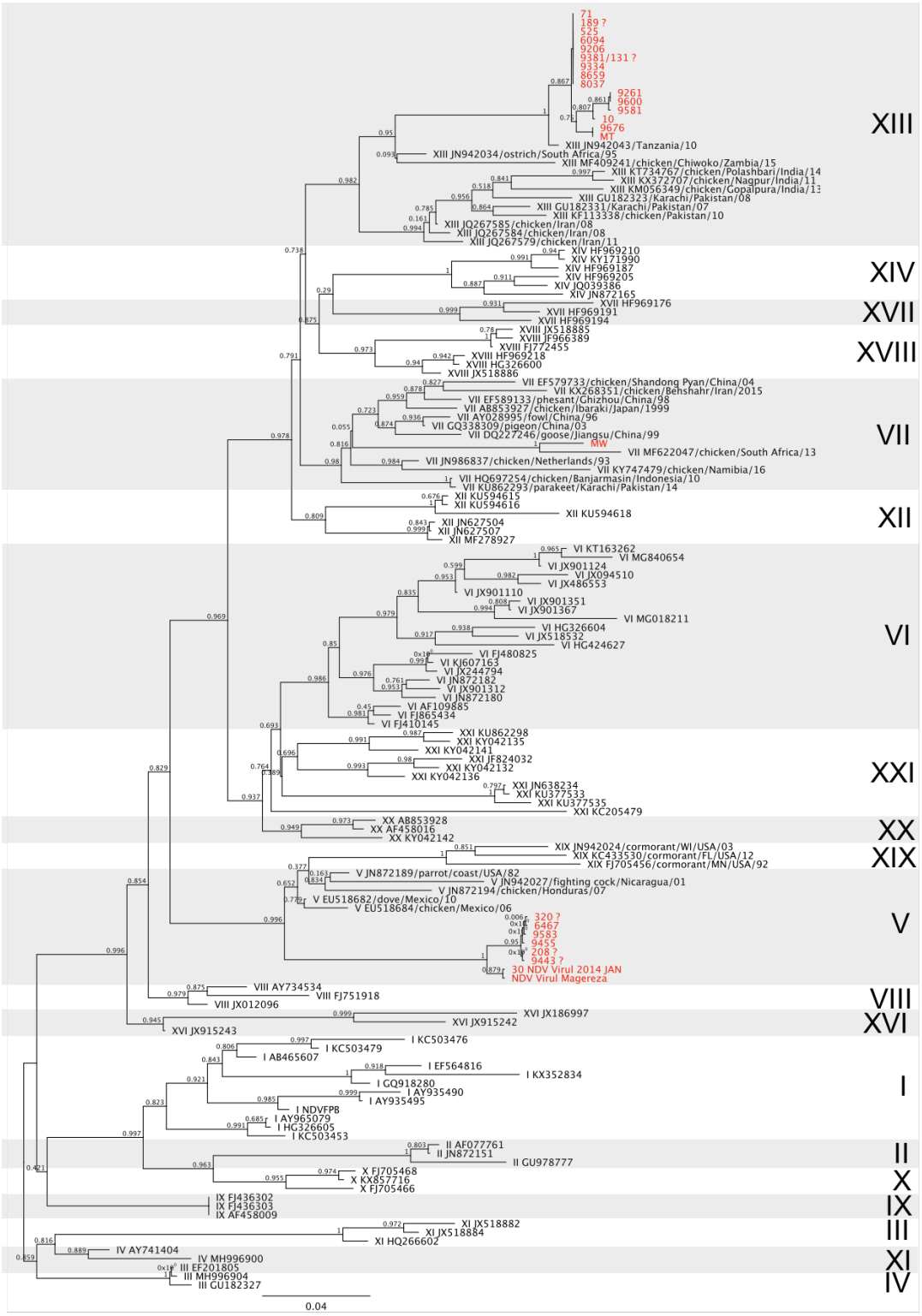
Figure 1. Phylogenetic analysis of the fusion protein gene sequences derived from MinION. The evolutionary history was determined by using Randomized Axelerated Maximum Likelihood (RAxML v8) based on the General Time Reversible (GTR) model, with 500 bootstrap replicates. The sequences from Tanzania are in red.



TURKEY MANAGEMENT, ESPECIALLY RELATED TO NON-CONVENTIONAL MANAGEMENT E.G. ORGANIC AND NON-GMO PRODUCTION

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SUMMARY

The turkey of today, is a fast growing bird. If allowed to express its full genetic potential a male turkey can gain close to 2.5 pounds a week. In order for a turkey to express its full genetic potential management must be at its best. Management will include having a knowledge of nutrition, bird behavior, environmental conditions and several other factors. The better a producer can manage these factors the closer the turkey can meet its full genetic potential.

Turkey management has come a long way. From its beginnings, it is reported that the Aztecs would catch wild turkeys and clip their wings so they could not fly. Now to our modern barns, which can have a large part of the barn controlled by a computer. Turkeys were once raised in barn yards, where their main purpose was to help control insects. As Thanksgiving gained popularity as a holiday, one that is all about food consumption, turkey became the meat of choice. This required turkey production to increase, however only seasonaly. These birds were grown out in pastures, allowed to roost on tree branches and were often herded to market similar to cattle drives of the old west.

Over the years, turkey management has progressed from outdoor pens to environmental controlled barns where conditions can be achieved allowing birds to reach their genetic potential. Turkey producers need to manage the air, feed and water inside these barns to be successful. Many of these can be controlled by a computer, called a controller box inside the barn. This controller box can send alarms to the producer when temperature, feed and water are out of the programed standards. The controller can raise and drop curtains, start feed lines and detect possible other problems in the barn. With this management technology one individual can manage a large number of birds without any help.

The modern-day consumer wants to know the food they consume is healthy and are raised in a humane manner. The turkey industry has responded to the consumer by producing antibiotic free, non-GMO and organic turkeys. Also, in response to the consumers' humane treatment, the industry has allowed independent auditors to come and document management practices. Most turkey companies have at least one and often several trained employees in animal welfare. An examination of the turkey industry would reveal that most companies are still producing a large number of conventional type birds however more and more companies are devoting more square footage to non-conventional type birds. Some companies produce only non-conventional birds. Although good management works for all bird types, producers raising non-conventional type birds need to step up their game and even get creative in their management practices.

Antibiotic free turkeys (ABF). ABF turkeys are birds that receive no antibiotics in the feed or in the water. Many turkey companies have all, their or a portion of their production as ABF. Companies may use different names for ABF: No Antibiotic Ever (NAE) and Raised Without Antibiotics (RWA) are some of the more common names. Managing ABF turkeys really means getting back to the basics of good management. Best management practices need to applied in managing the air, water, and feed. Creativity comes in generating downtime, stocking density and vaccination program.

Non-GMO turkeys (NGMO). NGMO turkeys are fed feed ingredients that have not been genetically modified. The management guidelines discussed above apply to NGMO birds. Feed management is critical, from feed manufacturing to feed delivery to the barn. If NGMO feed is being manufactured with other feed types, then a program needs to be in place to keep feed ingredients and mixing separate, as well as flushing between feed types. NGMO feed needs to be delivered to the correct barns.

Organic turkeys. Raising organic turkeys not only involves feeding only organic feed ingredients but it also involves having an organic system plan. The organic system plan outlines a production plan for raising organic birds in your production system. The organic system plan also outlines the approved products. One requirement for organic-raised birds is to have outside access. This puts a new level of management in a production system. Years ago, birds were moved indoors for disease control. Now these

non-conventional system birds are allowed outside. Management can be made more difficult since everything in the barn and in the outdoor access needs to be from organic or organic approved material. Gut health is essential in all these non-conventional birds but especially in organic birds because of their outdoor access.

Global Animal Partnership (GAP). GAP is an independent animal welfare rating system that was developed in conjunction with Whole Foods in 2008. GAP is a five-step rating program. Each of the five steps have a set of management criteria that needs to be met in order to obtain the appropriate step level. These management criteria include criteria like outside access, stocking density, having enrichments in the barn and in the outside access, meeting specific mortality numbers, and obtaining specific foot pad and lameness scores are just some of the criteria. GAP limits the growth rate and what services can be provided for the poult at the hatchery. All of this requires a great deal of record keeping which adds another level of management

There have been many articles and meetings discussing managing birds being raised as ABF, NGMO or ORG. Each have offered their own set of management practices that is necessary to be successful. Combining their recommendations, the top ten most often discussed are:

1. Downtime: The clock for downtime starts once the barn has been clean and is dry
2. Decreasing bird density: A reduction in density and increase in downtime may affect the income of the grower. A balance needs to be found between the two
3. Explore alternative products: Over the years several natural products (pre and probiotics, yeast, essential oils are examples) have been developed and improved to be used both in the feed and water. Be open to explore and see what products work best in your production system. ORG birds can only use products on their approved organic system plan list.
4. Improve brooder management: This includes starting birds in a clean environment, having good water sanitation, follow proper temperature guidelines.
5. Litter management: There needs to be a step up in managing litter, good tilling program and keeping the litter dry.
6. Feed intake post hatch: It is critical that the birds get on feed as soon as possible after hatch. Never run birds out of feed. Make sure only good feed ingredients are used to manufacture feed.
7. Reduce stress: Examine your production system and look for potential areas of stress and try to eliminate all the stress you can.
8. Biosecurity: In any management system biosecurity is important. However, it becomes even more important in a non-conventional management system.
9. Pay attention to details: This is a good practice in any management system. It is critical to know what is going on in your barns and what is happening with your birds. A good deal of stress can be reduced if a grower is paying attention.
10. Managing vaccination program: Better oversight of the coccidiosis and necrotic enteritis programs.

Non-conventional turkeys are here to stay at some level, so it is essential that producers learn to successfully grow turkeys in these new systems.

A CASE REPORT OF HIGH MORTALITY IN CHICKENS DUE TO INFECTIOUS BRONCHITIS VIRUS

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SUMMARY

In March 2019, the California Animal Health and Food Safety Laboratory (CAHFS)-Turlock branch received two submissions of broiler chickens from commercial flocks reporting increased mortality. Submissions consisted of both white and brown broilers. At necropsy, moderate to severely enlarged and pale kidneys were observed, with gross lesions indicative of dehydration. Microscopically, renal tubules were degenerated and distended with necrotic debris. Infectious bronchitis virus (IBV) was isolated and identified by qRT-PCR from kidney tissue pools and tracheal swab pools from both cases. Partial sequencing of the S1 hypervariable region was most similar to a local California variant. The outbreak lasted roughly one week in both flocks, with 2% total mortality in the brown broilers and 20% total mortality in the white broilers. Nephropathogenic IBV has been sporadically reported in California broiler flocks, and represents a significant pathogen due to the potential for high flock mortality.

INTRODUCTION

Infectious bronchitis is an acute highly contagious upper respiratory disease of chickens caused by infectious bronchitis virus; a gammacoronavirus in the family Coronaviridae (1). The viral genome is composed of single-stranded positive sense RNA and is subject to high mutation rates and recombination events, which results in the continual emergence of new IBV variants (2). Spike (S), envelope, membrane, and nucleocapsid are structural proteins in the IBV virion (1). Of these membrane proteins, S glycoprotein has been a popular target for molecular analysis due to its involvement in host cell attachment, membrane fusion, and viral entry to the cell. Serotyping of IBV isolates has been practiced traditionally; however, most laboratories currently utilize genotyping for IBV characterization (3).

CASE REPORT

On March 7, 2019, four live and three dead seven-week-old brown broiler chickens were submitted to the CAHFS-Turlock branch for diagnostic evaluation due to high flock mortality. The field technician reported no signs of depression in the affected flock. The mortality was high for approximately one week, with a peak daily mortality of 80 chickens and a total mortality of 420 chickens (2%) at the end of the outbreak in the affected house.

On March 8, 2019, eight live and one dead four-week-old white broiler chickens from the same company were submitted to the CAHFS-Turlock branch due to high mortality. The field technician observed depression and ruffled feathers in the affected chickens. High mortality continued for one week, with a peak daily mortality of 800 chickens, after which the chickens were sent for early processing. The total mortality in the week prior to processing was 4,600 chickens (20%) in the affected house.

Gross lesions observed at necropsy consisted of moderate to severely enlarged and pale kidneys with an enhanced tubular pattern, and urate accumulation in ureters. Microscopically, renal tubules were degenerated and distended with necrotic debris and tubular casts. The kidney parenchyma contained interstitial edema and mononuclear inflammatory cell infiltrates. Positive staining for IBV IHC was observed in kidney, trachea, air sac, bursa, and intestine sections in the white broilers; and in the kidney and intestines in the brown broilers.

Infectious bronchitis virus was isolated from kidney tissue pools from the white broiler submission and brown broiler submission. Partial IBV S gene sequences were obtained from kidney samples. Phylogenetic analysis of the sequences resulted in high sequence homology to CA 1737.

DISCUSSION

The isolated nephropathogenic IBV strains from the white and brown broiler chickens described in this report were most similar to CA 1737. CA 1737 was first isolated in 2003, and has not been previously reported in association with renal lesions (4). Many different types, subtypes, and variants of IBV exist and are continually being produced due to the rapid replication rate, high mutation rate, and genomic recombination events of IBV (5).

IBV is typically considered to be an upper respiratory pathogen, with the S1 protein being directly involved in binding of IBV to host cells (6,7). The S1 protein of respiratory IBV strains has a strong affinity for upper respiratory tract cilia and goblet cells, binding to alpha-2,3-linked sialic acids (6,8). Nephropathogenic strains have reduced affinity to respiratory tract cells and a high affinity for kidney cells, and have been shown to bind to a different sialic acid receptor (6,8).

The difference in mortality between the brown and white broilers in our study is an example of the importance of genetics in relation to the severity of a nephropathogenic IBV outbreak. The role of individual bird genetics in susceptibility to nephropathogenic IBV has been previously studied. An increased susceptibility in particular strains of specific pathogen free chickens, likely due to differences in immune responses to viral infection, was observed (9).

(The full length article will be published in *Avian Diseases*.)

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APPLYING COMPUTATIONAL SOCIAL SCIENCE TO THE VND OUTBREAK IN CALIFORNIA AND BEYOND

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SUMMARY

Backyard poultry (BYP) ownership is increasing in California and nationally. However, the location and number of BYP flocks remains unclear (2). Additionally, survey and anecdotal observations indicate that BYP owners have relatively poor biosecurity practices. Furthermore, California's limited resources to educate BYP owners has made it difficult to communicate with them in a coordinated fashion. This combination of poor biosecurity practices (e.g. exposure to wild birds) and unregulated bird movement can help facilitate the spread of disease among BYP flocks as evidenced by the 2003 and 2018 virulent Newcastle disease (vND) outbreaks in Southern California, both of which have been linked to backyard exhibition birds (1, 3). In an effort to better understand the risk of live bird movement and improve collaboration and biosecurity compliance among backyard poultry flocks, the UC Davis School of Veterinary Medicine-Cooperative Extension Poultry lab is utilizing novel computational social science tools and techniques for outreach and Susceptible-Infectious-Recovered (SIR) modeling related to vND. More specifically, we are using a new multi-lingual phone app in addition to social network analysis (SNA) and computational linguistics to 1) strengthen the network between BYP owners and outreach professionals, and 2) the development of SIR based disease models presented as an app in order to better inform stakeholders regarding disease spread and control. The development of these interactive and dynamic tools are a novel approach toward controlling vND in California.

INTRODUCTION

This manuscript will focus on preliminary results from YouTube.com. We studied a network of YouTube videos related to biosecurity for backyard poultry in order to gauge user engagement and consumption of BYP videos. The goal is to understand the web ecology of information and to identify what motivates user participation and consumption of YouTube videos for our outreach efforts.

MATERIALS AND METHODS

Collecting videos and attributes. YouTube videos from searching "biosecurity for backyard chickens (https://www.youtube.com/results?search_query=biosecurity+for+backyard+chickens)" were webscraped using Google Chrome's extension, Web Scraper. Videos from the initial search are referred to as "featured" videos. Videos that were suggested as videos to watch next were also scraped and are referred to as "recommended" videos. Videos from formal extension sources such as universities are referred to as "extension formal" versus videos from informal extension sources such as backyard poultry owners are referred to as "extension informal." Government affiliated channels such as the United States Department of Agriculture and the Centers for Disease Control are labeled as "government." Videos produced by farms, magazines and feed stores are labeled as "farm", "magazine" and "feed store," respectively.

Evaluating user engagement. The total number of times a video has been viewed as reported by YouTube was used as a measure of user consumption. Likes and dislikes were used as a measure of user participation with likes representing appreciation of content and dislikes representing disapproval of content.

Network analysis. An edge list was constructed from the data collected via webscraping and uploaded to Gephi a network analysis software.

RESULTS

The network consisted of 282 YouTube videos and 248 directed links with a resulting network density (directed) of 0.004. The average total degree centrality was 2.5 and both the average in- and out-degree centrality were 1.2. There are three components (one large and two smaller clusters) as seen in Figure 1.

DISCUSSION

Based on Figure 1, there are a few government and formal extension-type videos at the forefront with

12 out of the 20 featured videos falling under those categories. However, in terms of social media engagement, it seems that users are not consuming content related to backyard poultry from formal extension and government resources as much as they could. For example, the number of views of featured videos from those sources ranged from 40 to 75,206 views (median = 1,003) (Table 1). Furthermore, it seems that participation or interaction with videos is also very low with only a few people liking (less than 180) or disliking (less than 50) a featured video. From an outreach perspective, it seems that there is room for improvement and looking further into ways to improve public engagement via YouTube videos will be investigated. While this is just a snapshot representation of a single search at one point in time, these preliminary results provide some understanding about the type of information available to backyard poultry enthusiasts and provides insights about consumption and engagement patterns.

ACKNOWLEDGEMENT

This work is supported by the California Department of Food and Agriculture.

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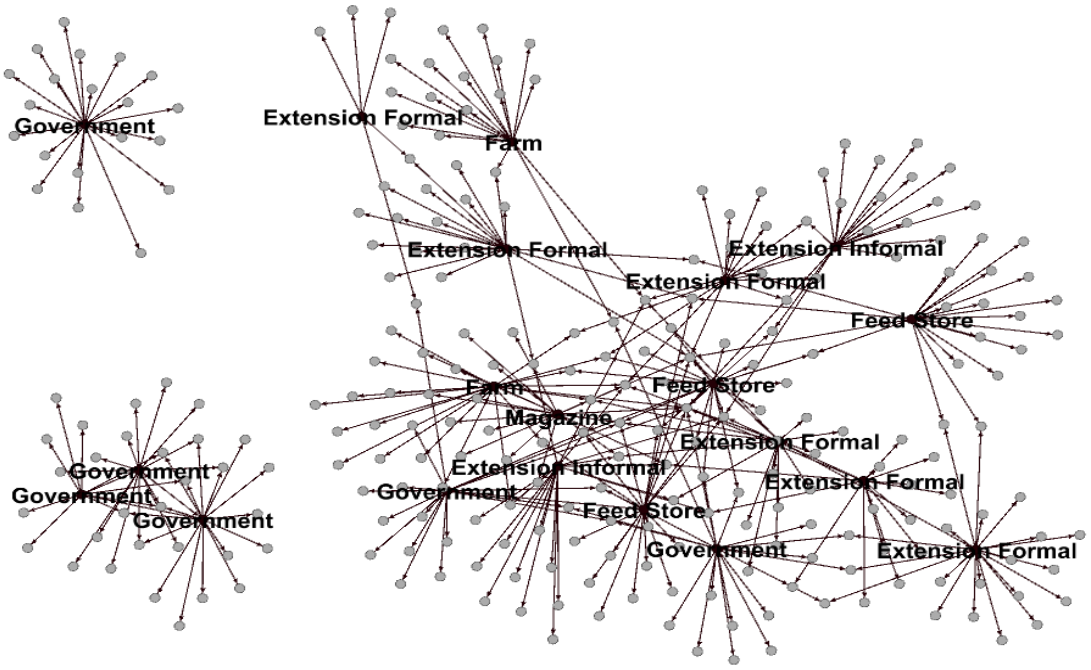
Table 1. Views, likes and dislikes for featured videos.

Video Name	Views	Likes	Dislikes	Source
Safety for your chickens - biosecurity	8	0	0	Extension Informal
Biosecurity for transporting livestock and poultry for owners	40	0	0	Extension Formal
4H poultry club workshop on biosecurity & NPIP	215	14	0	Farm
Preventing disease in backyard flocks	290	10	0	Extension Formal
How to make a DIY biosecurity kit	326	0	0	Magazine
Quarantining new chickens & practicing bio-security / why & how	334	5	0	Extension Informal
Biosecurity for pastured and organic poultry	400	8	0	Extension Formal
Poultry biosecurity - for urban and backyard bird owners	541	1	0	Extension Formal
Biosecurity for backyard chickens	559	14	0	Feed Store
Backyard biosecurity - Dr. Nancy Barr, MDARD	678	2	0	Government
Spotlight on: Biosecurity for birds	1,003	4	0	Government
Protect backyard chickens from Avian Flu	2,392	0	0	Extension Formal
Poultry biosecurity	2,998	28	1	Extension Formal
Biosecurity tips: How to prevent backyard chicken diseases	3,047	8	1	Feed Store
Biosecurity tips for backyard chicken-keepers	3,345	18	0	Government
Got a backyard flock? Here is how to prevent Salmonella	4,011	24	3	Government
Poultry bio-security: Basic tips to keep you and your flock healthy	4,241	19	2	Feed Store
Biosecurity for birds video with Hmong subtitles	9,491	9	6	Government
Biosecurity and health management on chicken farms	23,778	71	9	Farm
Simple steps to keep backyard poultry healthy	75,206	178	42	Government

Figure 1. Network of YouTube videos (featured= black nodes, recommended = gray nodes) from searching "biosecurity for backyard chickens" with nodes representing YouTube videos and links representing a recommendation was made.



OBTAINING THE MOST BENEFIT FROM AN ANTICOCCIDIAL SENSITIVITY TEST (AST)

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SUMMARY

The poultry industry has been using the same anticoccidial drugs for the prevention of coccidiosis for several decades. The last approval of a new anticoccidial compound in the United States by the Food and Drug Administration was in 1999 and currently no animal health company is actively working on developing new ones. In addition, in the United States, anticoccidials of the polyether ionophore class are considered antimicrobials and therefore cannot be used in the production of poultry raised without antibiotics like the ones bearing the labels "No Antibiotics Ever" (NAE) or "Raised Without Antibiotics" (RWA). The production of poultry raised without antibiotics has skyrocketed in recent years due to concerns about the potential for transfer of antimicrobial resistance from poultry products to humans, relentless pressure from activist groups, consumer preferences and marketing campaigns. This has put the poultry industry in a difficult position as coccidiosis prevention must be achieved relying exclusively on the use of chemically-synthetized anticoccidials, the so-called "chemicals" and live vaccines. Although with a few exceptions like nicarbazin that has been used successfully every year since 1955, chemically-synthetized anticoccidials are more prone to the development of resistance, and when resistance develops, control of coccidiosis becomes more difficult.

Coccidiosis control is an important factor in the prevention of necrotic enteritis (NE), which has emerged as a significant disease in poultry raised without antibiotics (1, 2). This may be due to partial resistance to chemical anticoccidials coupled with lack of antibiotic effect from chemical anticoccidials and no use of antimicrobials with good anticlostridial activity and ionophores. On the other hand, the use of live vaccines has also increased significantly in recent years paralleling the increase in the production of poultry raised without antibiotics. Even though live vaccines are effective when properly administered, they have to cycle several times through the intestines in order to produce immunity. As a result, mild intestinal lesions are produced and since no antibiotics with anticlostridial activity can be used in raised without antibiotics production; the use of live vaccines without antibiotics has more than likely played a role as well in the emergence of NE as a significant disease in poultry raised without antibiotics.

Anticoccidial sensitivity tests (ASTs) have been developed and used to predict the efficacy of anticoccidial drugs in the field (3, 4, 5). These tests can be very useful when properly conducted and interpreted. With the current trends in poultry production, ASTs are one of the most valuable tools available to producers to manage coccidiosis control successfully in the long term.

INTRODUCTION

What is an AST? An AST is a laboratory test in which coccidia isolated from field samples (usually litter or droppings) from different farms from a production complex are tested under controlled conditions for sensitivity to various anticoccidial drugs with the purpose of predicting their efficacy in the field.

Why are ASTs important? In order to optimize the selection of prophylactic medication in the field, knowledge about the sensitivity profile of the parasites in question is essential. AST is the best method currently available to assess and predict the efficacy of anticoccidial drugs against field isolates of *Eimeria* spp. in commercial broiler production. The importance of conducting ASTs for early detection of resistance or "reduced sensitivity" before flock productivity is adversely impacted has been emphasized by several researchers and field veterinarians (6, 7, 8, 9, 10, 11, 12, 13, 14, 15).

ASTs are also used to determine anticoccidial efficacy in drug approval studies. They are a useful predictor of anticoccidial efficacy when local *Eimeria* spp. isolates are used by a commercial production complex and they are also useful to detect early changes in sensitivity to anticoccidial drugs.

How is the sensitivity determined? Sensitivity can be determined in various ways, some have suggested by comparing differences in body weight gain between the treated group and the negative and positive controls (8), while others have suggested excretion of oocysts (3), an anticoccidial index (8) or

body weight gain and feed conversion (10). However, since anticoccidial drugs are designed to prevent coccidiosis, according to L.R. McDougald (4), comparing percent reductions in lesion scores between treated and negative and positive controls is the most important parameter to detect changes in sensitivity. Therefore, although body weight gain, feed conversion ratio and livability are recorded for their economic importance, in this presentation, percent reduction in lesion scores will be the parameter used to assess sensitivity of field isolates of coccidia.

SAMPLE COLLECTION AND SUBMISSION

Before collecting samples, it is important to determine what anticoccidial drugs and what dietary inclusion rates will be used in the ASTs so that the laboratory ensures that they have all the anticoccidial drugs needed within the expiration period. It is also a good idea to mix all the treatment diets ahead of time and conduct drug assays for each treatment that includes anticoccidial drugs before the start of the ASTs to ensure that anticoccidials were mixed properly.

A very important part of an AST is the collection and submission of samples to the laboratory. This is important to preserve the viability of the oocysts present in the sample and to have a truly representative sample of the *Eimeria* spp. present in the production complex where the samples were collected. Since it is not possible, or necessary, to sample every house in a production complex, a number of samples (between 5 – 10) will suffice. As stated by H.D. Chapman (7) "it is likely that the results for one farm will be similar to other farms within the same complex, providing the same drug programs have been employed."

Litter or droppings samples should be collected from at least six locations in a zig-zag pattern from the brooder end of the house. If litter is collected it should be collected from the upper one third of the litter and enough to fill a gallon container and if droppings are collected there should be at least 24 fresh droppings collected including intestinal and cecal droppings. To increase the probability of recovering enough oocysts to prepare the inocula from the first pass, samples should be collected from flocks that are between 21 and 28 days old.

The samples should be shipped to the laboratory where the ASTs will be conducted in a well-sealed cooler by overnight express delivery mail and a few ice packs should be included to prevent the samples from overheating during transport. Samples should be prevented from overheating but not allowed to freeze as both excessive heat and cold are detrimental to oocyst survivability.

BRIEF DESCRIPTION OF THE TEST

Inoculum. Upon reception of litter or dropping samples, these are mixed with feed and fed to coccidia-free broiler chicks kept in cages with wire floors. Droppings from these birds are collected from days 4 – 7 and the oocysts recovered, cleaned, sporulated, and titrated (~ 10^5 sporulated oocysts/mL). Once prepared the inoculum is stored under appropriate conditions until the day of the challenge.

AST. On the first day of the test, chicks are received from the hatchery and placed at random in coccidia-free floor pens with new shavings. They are fed a standard starter feed free of any anticoccidial compounds until day 12. On day 12, the chickens are assigned at random to each cage (usually eight/cage), and switched to their respective treatment diets. There are usually three to five replicates per treatment. The chickens assigned to the negative and positive controls continue receiving unmedicated feed while the treated birds are fed diets containing the anticoccidials at the dietary concentrations to be tested in each treatment. On day 14, all chickens except the negative control are infected with the inoculum previously prepared from the litter or droppings samples and titrated to contain approximately 10^5 sporulated oocysts of mixed *Eimeria* spp./mL. The inoculum is administered by oral gavage with a graduated syringe. The inoculum usually contains a mix of *Eimeria acervulina*, *E. maxima* and/or *E. tenella*. Also on day 14, all chickens are weighed by cage and the average weight per chicken calculated and used as the initial weight for the AST. At this time, the amount of feed administered to each cage is also recorded in order to calculate feed conversion ratio at the end of the test. On day 20, all the chickens in each cage are weighed again in order to calculate average body weight gain. Also on day 20, the remaining feed is weighed in order to calculate the feed conversion ratio for each treatment. At the same time, the chickens are humanely euthanized in order to remove the intestinal tracts and score the severity of the intestinal lesions induced by the coccidial challenge by the method of Johnson and Reid (16).

INTERPRETATION

Based on the manuscript by L.R. McDougald (4), if lesion scores for any given region of the intestinal tract (upper, middle, lower, or ceca) are reduced by 50% or more the isolate is considered sensitive to the respective anticoccidial. If lesion scores are reduced between 49 and 30%, the isolate is considered intermediate in sensitivity to the respective anticoccidial. If the lesion scores are reduced 29% or less, the isolate is considered resistant to the respective anticoccidial.

OTHER CONSIDERATIONS

Even though an AST only last 20 days, samples must be submitted and passed in chicks to prepare the inoculum and then chicks must be ordered ahead of time, feeds mixed and results summarized so in reality expecting results earlier than six weeks and more than likely eight weeks is not realistic. Ideally, a preliminary dose titration study should be conducted with the isolates in order to find the dose that will produce enough gross lesions and growth depression without mortality (this will add to the time in which results can be expected to be completed).

The most benefit from conducting ASTs is derived from testing samples from a complex at least once per year, over time, a reasonable assessment of efficacy and resistance can be produced and used to design more effective coccidiosis prevention programs in which chemotherapy is the main means of control. Likewise, for consistency it is better to use the same facility for ASTs. It is also important to submit representative samples ensuring they are not exposed to direct sunlight, excessive heat or cold and that they are shipped by express mail delivery to favor the survivability of the oocysts. Remember that having to perform a second pass is undesirable as the proportion of *Eimeria* species in the original sample can change and give a false assessment of sensitivity.

Submission forms must be filled out completely noting the previous anticoccidial use history and detailing what anticoccidial drugs and dosages are to be tested.

CONCLUSION

As the poultry industry grows a larger percentage of birds without ionophore anticoccodials and relies more on chemically-synthetized anticoccidials, ASTs can provide a very valuable tool to design more effective anticoccidial programs for the prevention and control of coccidiosis. Maintaining efficacy of anticoccidial drugs to prevent coccidiosis is important to minimize the incidence of necrotic enteritis which has emerged as a major disease in poultry raised without antimicrobials.

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EFFECT OF THE PHAGE COCKTAIL SALMOFREE® ON THE POULTRY MICROBIOME IN A COMMERCIAL FARM

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SUMMARY

Sciphage® is a spin-off of the University of Andes in Colombia, focused on the development of products that help to reduce the use of antibiotics in different industries. Our first product, a phage cocktail named SalmoFree®, addresses the poultry industry; this study, developed by Sciphage and the Universidad de los Andes, shows the beneficial effect of SalmoFree in the broilers. The microbiota of the broiler chicken gut affects health, metabolism and immunity of the chicken thus it has a positive impact on the animal productivity. In this study, 16S rRNA gene high-throughput Illumina sequencing was used to evaluate the effect of a SalmoFree in the microbiota of the cecum of broilers reared in an extensive farming system. Phages were incorporated in the broilers' drinking water using three doses at the grower stage.

RESULTS

Analyses of similarities among communities over time allowed us to identify two stages of microbiota development at the last stage of the production cycle. The core microbiome analyses of the composition of the microbiota identified some key species in the adaptation of the microbiota at the last stage of the production cycle. Among these there are some important degraders of complex polysaccharides and producers of short chain fatty acids (SCFA) such as *Eisenbergiella* and *Lachnoclostridium*. Additionally, the results showed that the phage cocktail did not affect the normal development of the microbiota structure while its application contributed to reduce the presence of pathogenic enterobacteria including *Salmonella*. Differential taxa analyses by treatment determined that *Campylobacter*, *Helicobacter*, *Butyricimonas*, and *Rikenellaceae* were either negatively or positively associated with the treatment. These results suggest that phages can contribute to improve the chicken health and reduce pathogens burden at the end of the production cycle of broilers. This study also presents a deeper understanding of the microbiome in broilers in a commercial scenario.

UPDATE ON IBD VIRUSES INFECTING BROILERS TODAY AND HOW INACTIVATED VACCINES PROTECT AGAINST DIFFERENT VIRUSES IN THE AL2 FAMILY

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INTRODUCTION

Infectious bursal disease virus (IBDV) is an extremely hardy double stranded RNA virus that can cause profound immune suppression—especially if infections occur prior to three weeks of age. This potential is high in the US because broilers are raised on built-up litter, resulting in a high infection pressure and competition between subtypes to override maternal antibodies and be the first to infect the next placement of birds. Not surprisingly, we see tremendous diversity of antigenic variant IBDVs in this country. This paper will summarize the findings and trends in IBDV isolations from broilers over the last several years as well as show how today's commercial 4-way IBD vaccines compare on challenge protection against today's most prevalent variant type, AL2, and its close relatives.

MATERIALS AND METHODS

Field Survey. Five to six bursas were collected from over three hundred broiler flocks between 2-5 weeks of age. RT-PCR analysis was performed to amplify the VP2 gene and positive samples were then submitted for VP2 sequencing. The 139 field IBDVs were divided into one of the following 5 variant categories: Del-E, AL2, T1, Group-6 and New-type. The relative prevalence of each major variant group is shown in Figure 1.

Challenge Study. 320 four-week old SPF leghorns were placed on fresh pine shavings into four different rooms. Four days later, 16 birds in each room were vaccinated with a partial dose of one of four different killed IBD 4-way vaccines while 16 birds were left unvaccinated. At four weeks after vaccination, one room remained unchallenged while all birds in the other rooms received 3.5 EID50 IBDV by eye/nose drop of either the AL2 prototype challenge virus or one of the two most common AL2-like viruses AL2b or AL2c (see Peak B differences in Figure 2). 10 days later, bursa to body weight ratios (B:BW) were determined to calculate mean B:BWs and percent protection by vaccine treatment. Challenged birds with a B:BW within two standard deviations of the mean B:BW of their respective non-challenged controls were considered protected.

RESULTS

The ranking of virus types by prevalence was AL2 family (52%), T1 (17%), New-type (15%), Del-E (8%) and Group-6 (8%). There were 4 unique AL2 subtypes with the original prototype still comprising the vast majority (82%). There were 3 subtypes of T1 virus, 5 subtypes of Group-6 and 4 subtypes of New-type—sharing unique mutations of Del-E/GLS, Del-E/AL2, Del-E/Group-6 and Classic/Variant. Compared to the 2009-11 survey (1), AL2 viruses remained the most prevalent type—again making up about half of all field viruses. T1 viruses were again the second most common type recovered. New-types saw a big jump from 3% to 15%, while Del-E types fell from 21% to 8%.

In the challenge study, Vaccine A gave over 90% protection against all three AL2 viruses. Vaccine B gave over 90% protection against 2 of the 3 AL2 viruses. Vaccines C and D gave significantly lower protection (6-63%) against the three AL2 viruses, with both vaccines averaging less than 50% protection.

DISCUSSION

The AL2 virus confirmed that it was "special" from the moment it was included in broiler progeny challenge studies on the Shore just over 20 years ago (2). Namely, broiler flocks that were well protected against Del-E challenge were often not protected against AL2. This was further bolstered by a fractional dose study comparing several commercial inactivated IBD/Reo vaccines that showed that AL2 was the only virus among a panel of variants that resulted in low protection levels in some of the vaccine treatments (3). The AL2 virus and its close relatives have been the most predominant IBD group in broilers for at least the past 10 years, making up about half of all field isolations (4). In addition, the AL2-like viruses have made up about 25% of the entire family in that same time period—prompting the question "how do today's

inactivated vaccines perform against AL2 and its most common sub-types?

The results demonstrated two key findings:

1) there were two tiers of protection that were significantly different (based on mean B:BWs), and

2) vaccines that protected well against AL2 also tended to protect well against the two AL-2 like viruses.

It is interesting to note that Vaccines A and B each contain a non-Delaware type variant IBDV. Perhaps the addition of non-Delaware variants in these two formulations contributed to antigenic diversity critical to improving cross protection against the AL2 family. It is also interesting that Vaccine B protected as well as Vaccine A against AL2 viruses containing the "GLS" mutation, 321-E, but was less protective against the AL2 subtype not containing that unique mutation (AL2-c).

In summary, this study reaffirms that commercial inactivated vaccines can vary significantly in their protection levels against the most prevalent AL2 family of viruses.

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Figure 1. Breakdown of 139 IBDVs From 2015-19 Field Surveys

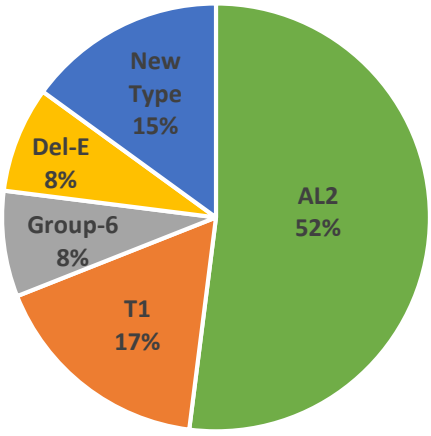
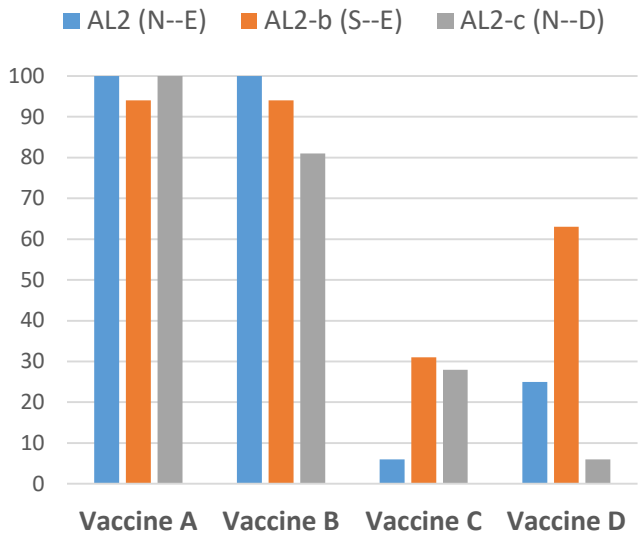


Figure 2. Protection Afforded by 4-way Vaccines Against AL2 Viruses



INFECTIOUS BRONCHITIS CHALLENGE STUDIES IN BROILERS—A COMPARISON OF IB VACCINATION PROGRAMS AND DMV/1639 PROTECTION

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INTRODUCTION

Infectious bronchitis (IB) variant DMV/1639 has become a growing challenge to the U.S. broiler industry, having first spread from Pennsylvania to Delmarva and then most recently to regions as widespread as Arkansas, the Southeast and Texas. To date, no commercial DMV/1639 vaccine is available so the industry has largely chosen to use various combinations of heterologous vaccine serotypes to attain cross protection. Because the commercially available GA08 vaccines have demonstrated the best levels of DMV/1639 protection in SPF leghorns (1), most broiler vaccination programs include GA08 plus 1-2 other vaccine serotypes to help control DMV/1639 challenge. A broiler study confirmed that the addition of either Mass, Ark or GA98 to GA08 did indeed improve DMV/1639 protection when given by eye drop (2). This paper will compare DMV/1639 protection in two subsequent broiler studies in which various bivalent and trivalent IB vaccine combinations were given by coarse spray.

MATERIALS AND METHODS

In each study, commercial broilers were spray vaccinated post hatch with various combinations of IB vaccines. Study 1 compared Mass+GA08, Mass+GA98, GA08+GA98 and Mass+GA08+GA98 to controls. Study 2 compared Mass+GA08+GA98 and Mass+Ark+GA98 to controls. Each vaccine treatment was allocated to five isolators of 18 birds each. At 25 days of age, three isolators per treatment (21 birds) were inoculated with 3.5 EID50 DMV/1639 virus by eye and nose drop. At 31 days of age, birds were examined for clinical signs and lesions and tracheas were swabbed for IBV-PCR and formalin fixed for histopathological evaluation.

RESULTS

The clinical signs and airsacculitis lesions in both challenge studies were modest and numerically but not significantly less prevalent in all vaccine treatments compared to controls. Nonetheless, the DMV/1639 challenge "take" was solid in both studies based on both histopathology and IBV-PCR. All vaccine treatments significantly reduced mucosal thickness (inflammation) and IBV-PCR loads in tracheas. Only the Mass+GA98 treatment did not give significant improvements over challenge controls on the incidence of Grade 3-4 lesion scores or Ct values >35 (see Table). The bivalent combinations containing GA08 in Study 1 gave similar protection, with Mass+GA08 slightly better on tracheal lesion scores and protection at the Ct-35 level and GA98+GA08 slightly better on mucosal thickness and protection at the Ct-30 level. The trivalent combination Mass+GA08+GA98 meanwhile consistently gave the best protection levels against tracheal lesions and IBV loads, with a 93% protection rate at the Ct-35 level. In Study 2, this same trivalent combination again performed on a high level, resulting in 67% protection at the Ct-35 standard. In comparison, the trivalent combination Mass+Ark+GA98, while performing comparably on tracheal lesions, did not measure up on reductions in IBV infection.

DISCUSSION

A previous study showed that adding a second IB vaccine serotype to GA08 significantly improved DMV/1639 protection in the following order of efficacy: Mass ≥ GA98 > Ark. In Study 1, here, the bivalent vaccine combination of Mass+GA08 was again slightly better than GA98+GA08 in helping reduce DMV/1639 infections. However, the combination of all three of these vaccines raised protection levels to over 90%. This Mass+GA08+GA98 combination again proved to be very effective in Study 2; however, swapping out Ark for GA08 to go with Mass+GA98 resulted in a significant drop in DMV/1639 protection—again demonstrating the importance of including the GA08 serotype for optimal protection levels.

Broiler IB vaccination programs are largely determined by the time of year and the type of IBVs present in a given geographical region. Where DMV/1639 viruses are in circulation, most complexes will include GA08 into their program—at least in the

higher-challenge winter months. These studies suggest that Mass is a marginally better choice than GA98 to combine with GA08. However, the presence of GA98 field challenge should factor into that bivalent choice. If the DMV/1639 infection pressure is rather high, the trivalent combination of Mass+GA08+GA98 should be seriously considered.

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Table 1. Histopathology and IBV-PCR results of vaccine treatments challenged with DMV/1639 in two studies.

Study	Vaccine Program	Histopathological Evaluation			IBV-PCR Ct Value Analysis		
		Grade 3-4 scores**	Mucosal thickness (nm)	Mucosal Protection	Mean Ct	>30 Ct (mod/no IBV)	>35 Ct (low/no IBV)
1	No Vaccination	100%a*	172.9a	13%a	24.5a	0%a	0%a
	Mass+GA08	33%bc	103.4b	60%b	33.5b	55%b	40%b
	Mass+GA98	67%ab	116.7b	44%b***	32.1b	74%bc	21%ab
	GA08+GA98	43%bc	91.0bc	76%bc	32.7b	71%bc	31%b
	Mass+GA08+GA98	14%c	69.7c	92%c	39.0c	100%c	93%c
2	Mass+GA08+GA98	29%b	82.5b	64%b	36.6b	100%b	67%c
	Mass+Ark+GA98	52%b	88.6b	52%b	34.2b	90%b	33%b
	No Vaccination	100%a	141.5a	15%a	27.5a	20%a	7%a

*Treatments within the same study and column not sharing a common letter are significantly different ($p \leq 0.05$).
**Grade 3 and 4 tracheal lesions of gland or cilia loss or lymphoid inflammation.
***Protection is nearly significantly lower than the GA08+GA98 treatment ($p \leq 0.08$).

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VACCINATION WITH A MODIFIED LIVE E. COLI VACCINE PROTECTS LAYERS AGAINST HOMOLOGOUS AND HETEROLOGOUS APEC STRAINS

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INTRODUCTION

Avian Pathogenic *Escherichia coli* (APEC) is one of the costliest poultry diseases affecting broilers, broiler breeders and layers. It is usually an opportunistic bacterial condition affecting birds exposed to stress, and respiratory and immunosuppressive diseases. Colibacillosis usually manifests as respiratory infections, peritonitis and/or septicemia that ultimately result in increased mortality and suboptimal egg production. Historically, APEC infections have been controlled with antibiotics. However, due to concerns about the development of multi-resistant bacteria and market restrictions, there has been a heightened interest in exploring E. coli vaccines that can result in a specific immune response against APEC. The most common APEC reported from field infections belong to O1, O2 and O78 serogroups (1). Considering this disease profile, the objective of this trial was to evaluate the effect of a live *E. coli* vaccine (LVEC) against homologous (O78) and heterologous (O2 – S strain) APEC strains in commercial laying hens.

MATERIALS AND METHODS

Two open sided research layer houses with two-tiered straight pullet and laying cages received 90 day-old Isa Brown layers each (3 birds placed per cage – 30 cages per house). Feed and water were made available as per rearing guide by Isa Brown commercial layers. Each of the houses was assigned to one of the two challenge ages (23 or 44 weeks) and each cage was assigned to a vaccine/APEC challenge treatment (Table 1).

Birds from T01, T02 and T05 treatment groups received a LVEC by coarse spray at 10 days and 12 weeks of age. At 23 and 44 weeks of age, birds from house A and house B, respectively, were challenged intratracheally with either O78 (1.25x109 CFU/bird), O2 (2.00x109 CFU/bird) or sham. Mortality was recorded daily, and at 7 days post-challenge, all birds were euthanized and scored for APEC lesions: airsacculitis (0 to 3), perihepatitis (0 to 2), pericarditis (0 to 2), cellulitis (0 or 1), salpingitis (0 to 3), and yolk peritonitis (0 or 1). For each treatment, comparative protective percentage (CPP) was calculated by adding all birds showing no lesions (airsacculitis, perihepatitis, and pericarditis) and dividing by the total number of birds. In addition, egg weight and egg production (cumulative egg production and hen house production) were evaluated for the House B birds (45 weeks).

The trial design was a completely randomized design considering each cage as the experimental unit and birds as the subsampling unit. All statistical analyses were conducted at a 0.05 level of significance.

RESULTS

House A (23-week challenge). At 24 weeks (7 days post-challenge), there was no mortality observed in the LVEC O78 challenged birds, while non-vaccinated birds had 7% (1/15) mortality. In the O2 challenged groups, non-vaccinated birds presented a 27% (4/15) mortality rate whereas LVEC birds presented at 7% (1/15). There was no mortality or any clinical signs in the non-challenged groups. By Kaplan-Meier survival analysis, it was confirmed that LVEC vaccination of birds resulted in significantly lower mortality (P=0.05). For the O78 challenged birds, the CPP of non-vaccinated and LVEC birds was 62% and 95%, respectively. The percent of birds with severe (2-3) APEC scores for airsacculitis, pericarditis, and perihepatitis of non-vaccinated birds was 100%, 80%, and 87%, respectively, whereas LVEC birds showed 7%, 0%, and 3%. For the O2 challenged birds, the CPP of non-vaccinated and LVEC birds was 47% and 87%, respectively. Non-vaccinated birds had 33%, 20%, and 27% severe (2-3) APEC scores for airsacculitis, pericarditis, and perihepatitis, respectively, while LVEC birds had 13%, 7%, and 7% for the same scores.

House B (44-week challenge). At 45 weeks (7 days post-challenge) for the O78 challenged groups, LVEC birds showed a mortality rate of 7% (1/15) while non-vaccinated birds had 20% (3/15) mortality.

In the O2 challenged groups, non-vaccinated birds presented a 33% (5/15) mortality rate whereas LVEC birds presented 20% (3/15). There was no mortality or any clinical signs in the non-challenged groups. For the O78 challenged birds, the CPP of non-vaccinated and LVEC birds was 49% and 97%, respectively. The percent of birds with severe (2-3) APEC scores for airsacculitis, pericarditis, and perihepatitis of non-vaccinated birds was 20%, 20%, and 13%, respectively, whereas LVEC birds had 0% for the 3 scores. For the O2 challenged birds, the CPP of non-vaccinated and LVEC birds was 49% and 89%, respectively. Non-vaccinated birds had 33%, 20%, and 33% of severe (2-3) APEC scores for airsacculitis, pericarditis, and perihepatitis, respectively, while LVEC birds had 13%, 7%, and 7% for the same scores. As for the production parameters at 45 weeks, the total egg production for non-vaccinated birds was 13,053 eggs (145 eggs/hen) while LVEC birds produced 14,038 eggs (156 eggs/hen). In addition, the egg weights of LVEC hens averaged 3.3 grams higher than eggs from non-vaccinated hens.

DISCUSSION

The two APEC challenge strains resulted in mild mortality and clinical signs in the non-vaccinated hens—an appropriate challenge methodology that resembles clinical outbreaks in the field. The differences in mortality and CPP between non-vaccinated and LVEC vaccinated laying hens support that vaccination can result in significant protection against homologous and heterologous APEC infections. It is well known that different APEC strains possess an array of various virulence factors (colonization factors, adhesins linked to fimbriae or not, invasion factors, mechanisms of lysis in the serum, iron acquisition systems, opsonization prevention, and toxins) that can make these bacteria extremely resistant to an immune response (2). Regardless, the LVEC vaccine was shown herein and in previous studies to result in effective protection against homologous and heterologous challenges. In a broiler study, Cookson *et al*. (3) reported lower prevalence and severity of airsaculits in LVEC vaccinated birds that were challenged intratracheally with heterologous APEC strains of O1, O2 and O18. Similar results were attained by La Ragione *et al*. (4) where LVEC vaccination significantly decreased mortality and colibacillosis lesions when birds were challenged with an O78 or untypeable APEC strain. As mentioned previously, the virulence factor diversity of the APEC strains creates challenges in terms of effective immune responses. Therefore, it is likely that the success of LVEC vaccines is related to its mucosal route of administration (4) and triggering of cellular immune responses (heterophils, macrophages and other antigenic presenting cells) that are important in the initial stages of controlling the infection.

In conclusion, vaccination of laying hens with LVEC by coarse spray resulted in effective protection against O78 and O2 APEC strains at 23 and 44 weeks of age while improving egg production performance.

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Table 1. Vaccine and Avian Pathogenic *Escherichia coli* challenge treatments administered to Isa Brown layers.

Treatment #	Vaccination	APEC challenge	Number of birds (cages) per treatment	
			House A (23-week challenge)	House B (44-week challenge)
T01	Yes	O78	15 (3)	15 (3)
T02	Yes	O2	15 (3)	15 (3)
T03	No	O78	15 (3)	15 (3)
T04	No	O2	15 (3)	15 (3)
T05	Yes	None	15 (3)	15 (3)
T06	No	None	15 (3)	15 (3)

THE INCREASING IMPORTANCE OF *SALMONELLA* INFANTIS AND HOW BROILER LIVE *SALMONELLA* TYPHIMURIUM VACCINATION CAN HELP MITIGATE THIS CHALLENGE

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INTRODUCTION

Recent microbiological testing data from the Food Safety and Inspection Service (FSIS) show that there has been an overall reduction of *Salmonella* prevalence in samples collected at poultry processing plants (1). From 2016/2018 to 2018/2019, there was a 38% and 36% reduction of percent positives of whole broiler carcass rinses and chicken parts samples, respectively. This improvement is in part related to the adaptation of the poultry industry to the new FSIS performance standards and testing methods enacted in 2016 along with the adoption of several strategies on the live side that aimed to reduce the levels of *Salmonella* spp. at the plant. Regardless of this improvement and measures taken, there have been a few *Salmonella* spp. serotypes of human concern that have gained higher importance over the last decade. The most paradigmatic example is *Salmonella* Infantis. As reported by the Centers for Disease Control and Prevention (CDC), from 2005 to 2016, *S.* Infantis moved from the 9th to 6th place as the serotype most frequently isolated from human illness cases (2). On a similar trend, *S.* Infantis was reported by the FSIS to go from 10th to 5th place as the serotype most isolated from raw chicken from 2002 to 2014 (3), respectively. Considering the increasing importance of *S.* Infantis, the objective of the following two trials was to evaluate the efficacy of a live *Salmonella* Typhimurium vaccine (LVST) against this serotype.

MATERIALS AND METHODS

Two broiler studies were conducted to evaluate the cross-protective effect of LVST against *S.* Infantis.

Study 1. Sixty-four broiler chicks were placed in an isolation room into two separate pens (32 birds per pen) at day of age. One of the pens received birds vaccinated with LVST by spray at hatch and boosted by gavage at 14 days whereas the other pen received birds not vaccinated (control group). At day 33, birds from the two pens were comingled in one big pen (64 birds total with 32 birds per treatment) and were challenged with *Salmonella* Infantis at a target dose of 109 CFU per bird. At 43 days (10 days post-challenge), all birds were terminated and cecae samples evaluated for *Salmonella* spp. load (enumeration by most probable number [MPN]) and liver/spleen evaluated for prevalence.

Study 2. One thousand two hundred day-old broiler chicks were placed into 48 pens (25 birds/pen). Half of the pens (24 pens) received birds vaccinated with LVST by spray at hatch and boosted by drinking water at 14 days whereas the other half had birds not vaccinated (control group). At day 31, 15 birds per pen were challenged by oral gavage with a *Salmonella* Infantis inoculum at a target dose of 108 CFU per bird. The remaining 10 birds in each pen were horizontally exposed to the *Salmonella* Infantis challenge shed by the directly challenged birds. At 41 days (10 days post-challenge), five birds directly and five birds horizontally challenged had the cecae and liver/spleen sampled for *Salmonella* spp. prevalence and load (MPN) evaluation.

All statistical analyses were conducted at a 0.05 level of significance using two-sided tests.

RESULTS

Study 1. Using the comingled pen challenge methodology, LVST resulted in effective ($P<0.001$) cecae load reduction. Birds that were vaccinated had a 1.7 log reduction in cecae *Salmonella* spp. numbers when compared with control birds. This effect corresponds to a 98% reduction in terms of MPN numbers. Both treatments had a 90% prevalence of *Salmonella* spp.in the organs.

Study 2. In the 48 pen grow-out trial, both direct and horizontally challenged birds from vaccinated pens had a significant ($P<0.05$) *Salmonella* spp. prevalence and load reduction in the liver/spleen samples when compared with control pens. LVST vaccination resulted in a 9% and 18% decrease of positives in the liver/spleen for the direct and horizontally challenged birds, respectively. The load reductions were 1.0 and 1.4 log, respectively—a

decrease that corresponds to a 90 and 96% reduction in terms of MPN numbers. The cecae load was also numerically reduced by LVST vaccinations with direct challenged birds presenting 0.12 lower log (25% reduction on MPN) and horizontally challenged 0.10 lower log (20% reduction). In addition, vaccinated pens resulted in a numerically 3 point lower adjusted FCR.

DISCUSSION

Vaccination of broilers with LVST vaccines has been a novel strategy for *Salmonella* spp. reduction at the processing plant, and it has gained considerable traction and success in recent years. In previous trials, LVST vaccination has been shown to have a cross protective effect against *S*. Enteritidis, *S*. Kentucky and *S*. Heidelberg isolates (4). Lillehoj *et al*. in a comprehensive review on host immunity to *Salmonella* emphasized that each serotype triggers different cellular and humoral immune responses (5). Despite these differences, it seems that LVST vaccination results in immune responses that can cross-protect against various serotypes (4). Nevertheless, there was a need to validate if these cross-protective immune responses would be effective against *Salmonella* Infantis. The results presented herein showed that LVST does have an effect on *Salmonella* Infantis by reducing the numbers of this food borne pathogen in both cecae and organs. The prevalence and load reduction of the *Salmonella* numbers in the organs is an especially important parameter since translocation from the intestines to internal organs has been a concern with ground poultry products (6). The mechanism of action of LVST is probably its greatest differentiator when compared with other *Salmonella* interventions. To the author's knowledge, LVST vaccines are the only intervention commercially available in broilers that not only help to control the load of *Salmonella* spp. in the gut but also affect *Salmonella* that eventually translocate from the intestines or enter the bird's body through a different exposure route (7).

S. Infantis has caused an increased number of human health episodes over the past years. In fact, in 2018, a *S*. Infantis outbreak was reported by CDC where 129 people were hospitalized across 32 states. After investigation, it was shown that the outbreak strain was genetically related to isolates identified by FSIS from 76 poultry processing plants (8). Considering the high importance of this serotype and the relative efficacy of LVST against it, it can be concluded that LVST vaccination of broilers should be considered as a foundational part of a poultry producer food safety program.

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EFFICACY OF TWO COMMERCIAL INFECTIOUS CORYZA VACCINES AFTER CHALLENGE WITH A SEROTYPE C *AVIBACTERIUM PARAGALLINARUM* STRAIN ISOLATED FROM OUTBREAKS IN LAYERS AND BROILERS IN CALIFORNIA

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SUMMARY

Infectious coryza is a bacterial upper respiratory disease caused by *Avibacterium paragallinarum*. In recent years, a significant increase in infectious coryza cases has been observed in California, affecting both broiler and layer chickens. The strains isolated in California have been classified as Kume serotype C-2. However, genotypic analysis using the hemagglutinin genes HMTp210 and hagA demonstrated high similarities to strains that belong to serotype C-1. The aim of this study was to compare the efficacy of two commercial vaccines after challenge with a wild-type serotype C strain of *A. paragallinarum* isolated in California. Commercial vaccine designated 'C1' is a tetravalent killed bacterin vaccine that contains serotype A, B and C-1 strains as well as a variant of serotype B; this vaccine is not commercially available in the US. The second commercial vaccine, designated 'C2', is a trivalent bacterin that contains serotype A, B and C-2 strains and is widely used in North America. Despite differences in the serotype C strains included in vaccines C1 and C2, our results indicate that both vaccines, if administered in two doses as recommended by the manufacturer, are able to induce appropriate protection and reduce bacterial shedding when compared with unvaccinated chickens.

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 serotypes 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 serotypes A-1 to -4, B1 and C-1 to -4 (2).

In recent years, a significant increase in infectious coryza cases have been observed in California, affecting both broiler and layer chickens. The California *A. paragallinarum* isolates have been classified as serotype C-2 (3). In an attempt to genotypically distinguish *A. paragallinarum* isolates using the hemagglutinin genes HMTp210 and HagA, the strains from California presented similarities to both C-1 and C-2 strains (4). Although antigenic and genotypic differences exist among C strains, C-1, C-2, C-3 and C-4 isolates supposedly provide good levels of cross protection (5). The aim of this study was to compare the efficacy of two commercial vaccines containing C-1 and C-2 strains after a challenge with a wild-type serotype C strain of *A. paragallinarum* isolated in California.

MATERIALS AND METHODS

Vaccines. Two commercial vaccines were used in this experiment. Vaccine C1 is a tetravalent bacterin that contains serotypes A (0083 strain), B (Spross strain) and C-1 (H18 strain) as well as a variant of serotype B (48 strain). This vaccine is not commercially available in the US. Vaccine C2 is a trivalent killed bacterin vaccine that contains serotypes A (0083 strain), B (Spross strain) and C-2

(Modesto strain) and is widely used in North America. Birds were inoculated via subcutaneous route in the inguinal fold as per the manufacturers' instructions. Birds vaccinated with one dose received the vaccine at 11 weeks of age. Birds vaccinated with two doses received the vaccine at 7 and 11 weeks of age.

Birds. A total of 180 specific pathogen-free (SPF) chickens were hatched and raised in BSL-2 rooms. The birds were divided into 6 groups: (G1) positive control (unvaccinated, challenged), (G2) one dose of vaccine C1, (G3) one dose of vaccine C2, (G4) two doses of vaccine C1, (G5) two doses of vaccine C2 and (G6) negative control (unvaccinated, unchallenged).

Experimental design. Choanal swabs were collected from all birds and tested for *A. paragallinarum* by qPCR before vaccination. At 7 weeks, groups G4 and G5 (two doses of C1 and C2, respectively) received the first dose of the vaccine. At 11 weeks, groups G2 and G3 received a single dose of vaccine (C1 and C2, respectively) and groups G4 and G5 received the second vaccine dose. The *A. paragallinarum* challenge was performed oculonasally with 3.5 x 10^4 CFU/bird in 200 µL. Choanal swabs were collected at 4 and 8 days post-infection (dpi) to assess bacterial load by qPCR. All birds were euthanized at 8 dpi. Clinical signs were evaluated daily throughout the experiment using the following scoring system: (-) no signs, (+) nasal or ocular exudate or mildly swollen sinuses, (++) nasal and/or ocular exudate and moderately swollen sinuses and (+++) nasal and ocular exudate, severe swollen sinuses and depression. An index was calculated using the following formula: (∑respiratory scores/number of birds x 3) x 100.

RESULTS AND DISCUSSION

All birds were negative for *A. paragallinarum* by qPCR prior to vaccination. Local post-vaccination tissue reaction was observed in 10-15% of all vaccinated birds (G2, G3, G4 and G5). Systemic lesions were not observed in any of the groups. Microscopically, mild to severe lymphocytic infiltration in sinuses was observed in all challenged birds (G1, G2, G3, G4 and G5). Microscopic scores were not statistically significant ($P>0.05$).

All vaccinated birds (G2, G3, G4 and G5) exhibited significantly less severe clinical signs than the positive control birds (G1, $P<0.05$). The greatest reduction in severity was observed in birds vaccinated with one and two doses of vaccine C2 (G3 and G5) and two doses of vaccine C1 (G4). Furthermore, G4 was statistically similar to the unvaccinated and unchallenged birds (G6) ($P>0.05$), which emphasizes the protective effect of vaccine C1.

At 4 dpi, the bacterial load in the choanal cleft was significantly reduced in G5 (two doses of vaccine C2, $P<0.05$) as shown in Figure 2A. At 8 dpi, a significant drop in viral load was observed in all vaccinated groups compared with the positive control birds (G1, $P<0.05$, Figure 2B). G3 (one dose of vaccine C2) did not reduce bacterial shedding as well as the other vaccinated groups ($P<0.05$) (Figure 2B).

Overall, providing either of the two commercial vaccines subcutaneously twice with a 4-week interval between the doses was effective in reducing clinical signs and bacterial shedding after the challenge with the wild-type C strain of *A. paragallinarum*. Interestingly, a single dose of vaccine C1 (G2) induced a significant reduction in bacterial shedding at 8 dpi (Figure 2A), which suggests that this wild-type C strain from California might be more antigenically related to C-1 than C-2 strains. Although serotyping is the gold standard for classification of *A. paragallinarum* strains, this laborious technique is restricted to few laboratories around the world, which makes classification difficult and expensive. Further investigation is necessary to develop new tools and techniques for a more practical classification of *A. paragallinarum* strains. Although genotyping seems like a promising classification method (4), additional analyses are necessary to determine what genes are potentially linked to immunogenicity.

Vaccines and vaccination techniques are essential in disease prevention and control. Choosing appropriate strains, route of application and properly training the vaccination crews are crucial factors for a successful preventative program. Ventilation, biosecurity and prevention of concomitant diseases also interfere in the effectiveness of the vaccination plan. A constant search for new tools and assessment of the currently available alternatives are necessary to prevent respiratory diseases reemerging as a consequence of changes in poultry management.

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This experiment is part of a publication in press at the *Avian Diseases* journal (Gallardo *et al*., 2020. Infectious coryza: Persistence, genotyping and vaccine testing).

Figure 1. Clinical sign indices in SPF chickens vaccinated with two different commercial vaccines, C1 and C2, with one or two doses. Different superscripts represent statistical significance ($P<0.05$).

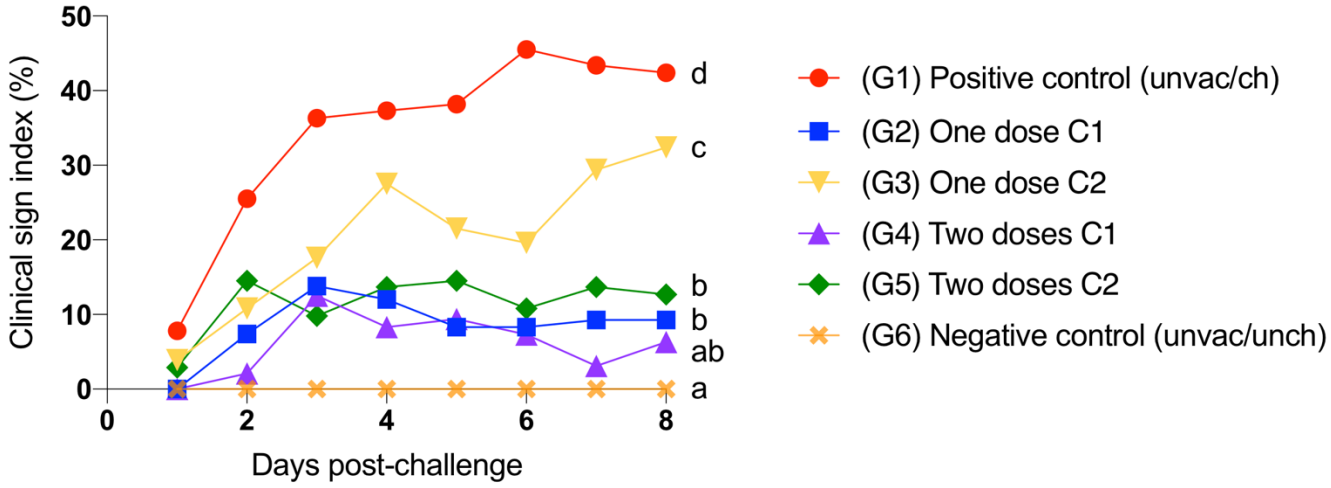
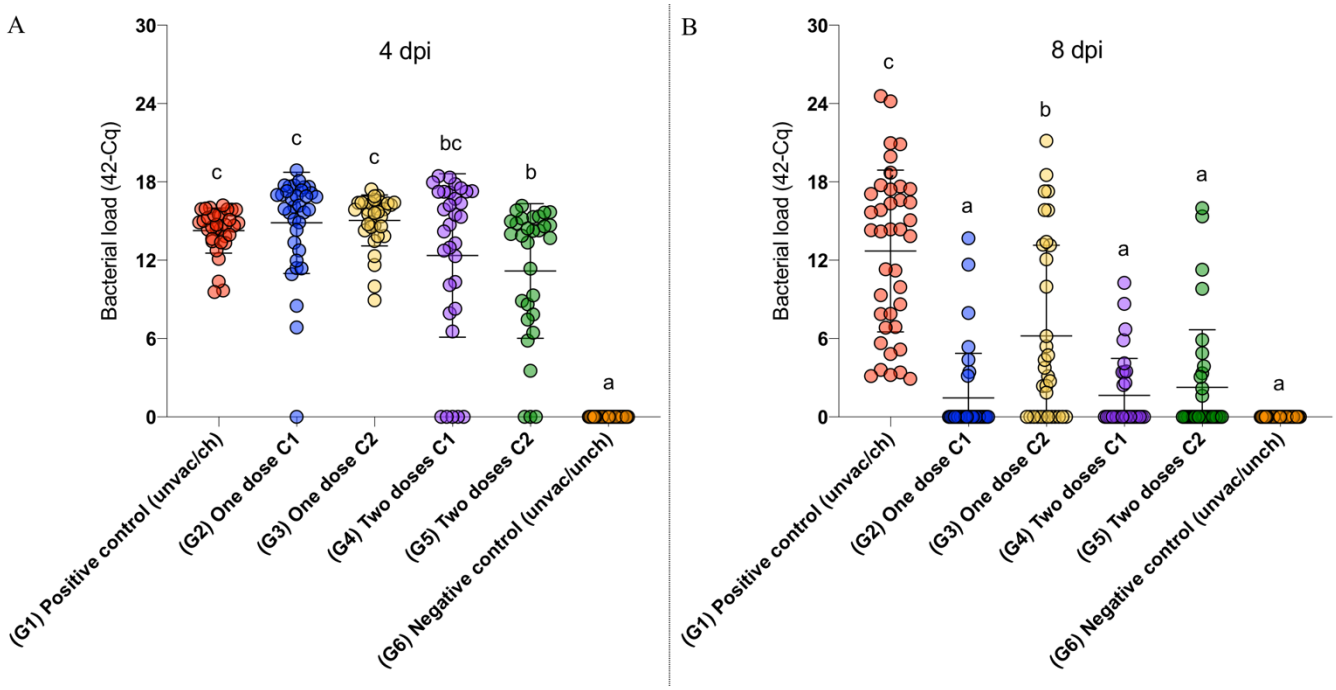


Figure 2. Bacterial shedding at 4- (A) and 8-days post-infection (B) in SPF chickens vaccinated with two different commercial vaccines, C1 and C2, with one or two doses. Different superscripts represent statistical significance ($P<0.05$).



PATHOGENIC CHARACTERIZATION OF AVIAN REOVIRUS VARIANTS STRAINS ISOLATED FROM CLINICAL CASES

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INTRODUCTION

Avian reovirus (ARV) is known as the main cause of viral arthritis and tenosynovitis in chickens and turkeys causing negative economic impacts in the poultry industry. Its double-stranded RNA genome divided into ten segments makes this virus prone to mutation and recombination events generating molecular variants (ARVv). Since 2003, new molecular variants of avian reovirus have been reported in different parts of the world1. Since 2011 some of these molecular variants have been causing outbreaks across the US (1,2,3,4,5).

In previous studies, we reported isolates of ARVv with homologies to S1133 below 77% based on sigma C (6,7). Despite the increased detection of ARVv, there is a lack of understanding about the pathobiology of these different variants. Therefore, the objective of the present study was to compare the pathobiology of selected ARV variants isolated from chicken hearts (H-strain) or tendons (T-strain).

MATERIAL AND METHODS

Four groups of one-day-old SPF chickens were placed in BSL2 rooms. Half of the chickens (n=28) in each group were challenged with 105 EID50 of the H-, T-, S-strain (vaccine S1133) or PBS as a negative control group subcutaneous via footpad. The other half of the chickens remained as contact birds. Body weights (Bw), Bw/hock joint thickness ratios (Bw-HJT), hearts, tendons, thymus and bursas were obtained weekly for four weeks. Viral loads in tendons and hearts were calculated by using real-time RT-PCR (8). Lymphocytic depletion in thymus and bursa was evaluated.

RESULTS AND DISCUSSION

Body weights and bw-HJT ratios (Fig.1) were lower in all inoculated groups compared with exposed groups starting at 3 DPC ($P<0.05$). At 28 DPC H- and T- exposed chicken weights were lower than the S strain inoculated chicken weights. S strain inoculated chicken weights were similar to the negative control chickens. Among the inoculated groups, the H- strain inoculated chickens showed the lowest weights. Bw-HJT ratios differences ($p<0.05$) among inoculated groups were observed between 8 and 21 DPC, being the H-strain inoculated group lower than T and T lower than the S- strain inoculated group. The Bw-HJT ratio was more sensitive to detect clinical effects of the compared viruses. Differences in viral load in tendons were at all times post challenge (Fig.2). While tendons from H- and S-strain exposed chickens did not have comparable viral loads to tendons from inoculated chicken groups, t-strain exposed chicken tendons had viral loads comparable to inoculated groups. This result suggest that T-strain ARVv is effectively transmitted horizontally, compared to the other two strains tested. The S- strain showed higher viral loads and less pathology in the studied tissues than the H- and T- strains, suggesting that the S- strain is better adapted to chickens. Viral loads in hearts showed a different pattern than in tendons being higher in the beginning and decreasing by the end of the experiment.

In conclusion, ARVv are not only genetically but also phenotypically different that conventional (S1133) strains.

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

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Figure 1. Body weight /hock joint thickness ratios (g/mm) from 1 to 28 days post challenge. Dashed lines show negative control and exposed groups. Continuous lines show H, T and S inoculated groups.

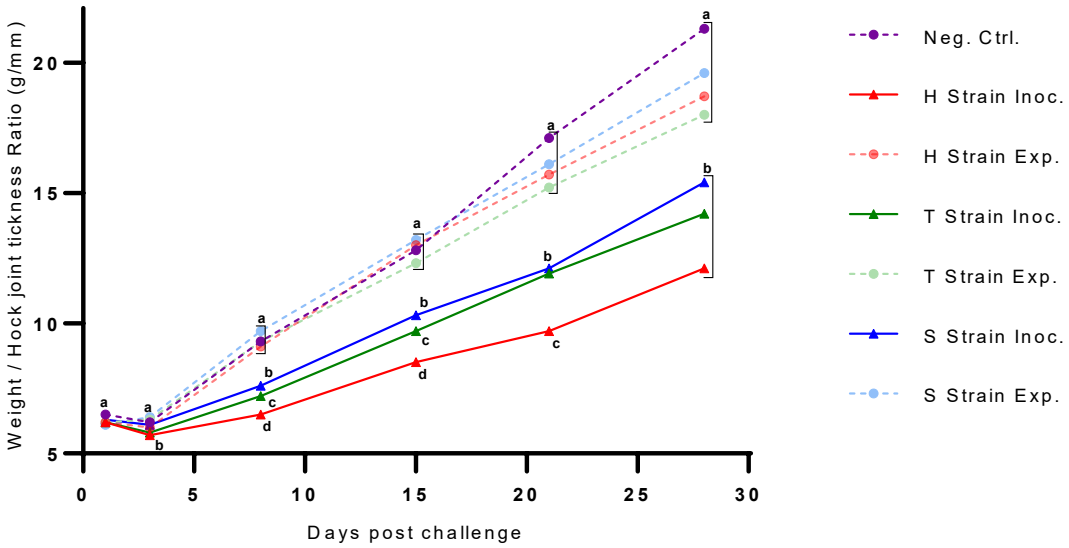
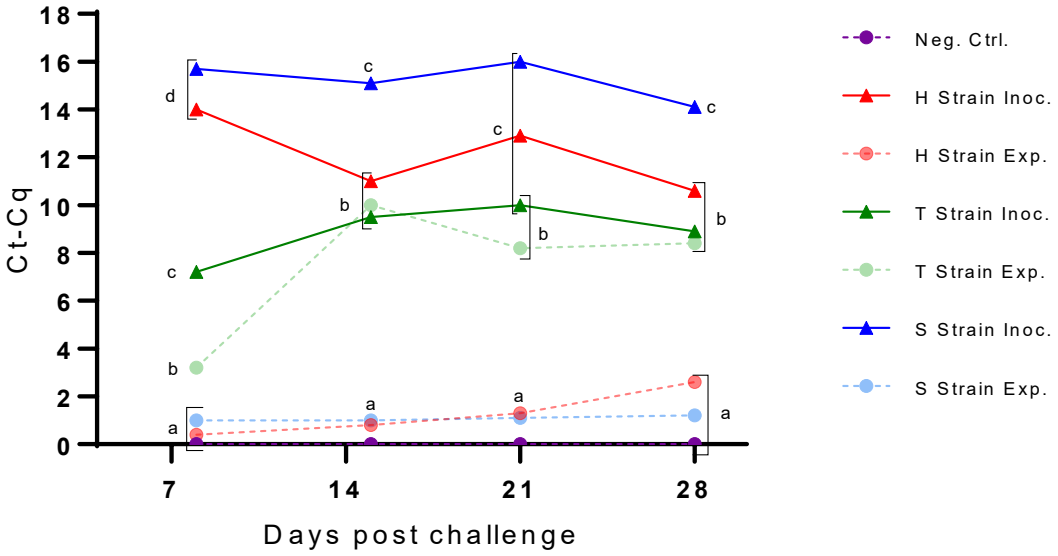


Figure 2. Viral loads (Ct-Cq) in tendons at 7, 14, 21 and 28 days post challenge. Dashed lines show negative control and exposed groups. Continuous lines are show H, T and S inoculated groups.



THE EFFECT OF INFECTIOUS BRONCHITIS VIRUS LIVE VACCINE ADMINISTRATION ON *MYCOPLASMA SYNOVIAE* INFECTION

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SUMMARY

Mycoplasma synoviae, the etiologic agent of infectious synovitis of chickens and turkeys, has remained a major concern for the poultry industry due to economic losses as the result of subclinical effects (such as poor feed conversion) and other complications such as airsacculitis and tenosynovitis. Manifestation of *M. synoviae* infection as an upper respiratory subclinical infection could be more complicated with co-infections such as Newcastle disease or infectious bronchitis. The aim of the current study was to evaluate the effects of a common infectious bronchitis virus (IBV) vaccine, Arkansas strain, when administered prior or concurrently to *M. synoviae* infection. In this trial, ninety-six SPF chickens were divided into eight groups and vaccinated at two, four, or eight weeks of age via eyedrop with a commercially available IBV vaccine. *M. synoviae* was administered at eight weeks of age and the groups were evaluated at 10 days post *M. synoviae* infection by gross lesion air sac lesion scoring, histopathological evaluation of tracheal lesions, serology, and real-time quantitative PCR. The results indicated that IBV vaccination impacted the gross and microscopic lesions evaluated as well as replication of *M. synoviae* in the tracheas of infected birds.

INTRODUCTION

Mycoplasma synoviae and infectious bronchitis virus (IBV) can cause tremendous economic losses due to mortality, condemnations, cost of treatment and vaccination, as well as the cost of control and monitoring to the commercial poultry industry. The losses may be much more noticeable and severe if both pathogens are present together in the commercial poultry. Previous studies showed that infectious bronchitis virus (IBV) vaccination in *M. synoviae*-infected broilers may lead to significant increases in airsacculitis and mortality compared to non-infected broilers (1-4), and *M. synoviae* infection may also affect IBV vaccine efficacy (5). Therefore, the aim of the current study was to evaluate the effects of a common IBV vaccine, Arkansas strain, when administered prior or concurrently to *M. synoviae* infection.

MATERIALS AND METHODS

In the current trial, ninety-six SPF leghorn-type chickens were divided into eight groups and vaccinated at two, four, or eight weeks of age via eyedrop with a commercially available IBV vaccine, Arkansas strain (Table 1). Then, all birds were screened at 14 days of age to confirm they were *Mycoplasma* free before inoculating with a pathogenic *M. synoviae* strain (K6677). Three IBV-vaccinated groups, along with a non-vaccinated control group, were inoculated with a virulent *M. synoviae* strain K6677 via aerosol at eight weeks of age. At 10 days post *M. synoviae* infection, the groups were necropsied and evaluated by air sac lesion scoring, histopathological evaluation of tracheal lesions, serology, and real-time PCR.

RESULTS AND CONCLUSION

The highest mean air sac lesion score and the highest mean tracheal mucosa measurement was observed in the group vaccinated with IBV at eight WOA and inoculated with *M. synoviae* (also at eight WOA). All of the groups inoculated with K6677 had mean air sac scores that were significantly higher than the non-*M. synoviae*-inoculated groups (all of which had no air sac lesions) ($P < 0.05$). The highest mean (genome) copy number log 10 (MCNlog10) of *M. synoviae* was detected in the group vaccinated with IBV at eight WOA and inoculated with *M. synoviae* at eight WOA; the *M. synoviae* replication in this group was significantly higher than the other *M. synoviae*-inoculated groups and the non-*M. synoviae* infected birds ($P < 0.05$). Very few footpad lesions were observed and there were no significant differences in mean score among the groups ($P < 0.05$). Although further results are pending, we may conclude that IBV vaccination may lead to the exacerbation of respiratory disease (including airsacculitis) in *M. synoviae*-infected chickens.

(Note: The full-length article will be published in *Avian Diseases*.)

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Table 1. Experimental design of the study. IBV: infectious bronchitis virus, K6677: a pathogenic *Mycoplasma synoviae* strain; WOA: weeks of age

Treatment	Infectious bronchitis virus vaccine (Arkansas strain)	Challenge with (K6677) *Mycoplasma synoviae* (8 WOA)	Number of Birds/Treatment
1	Yes – 2 WOA	No	12
2	Yes – 2 WOA	Yes	12
3	Yes – 4 WOA	No	12
4	Yes – 4 WOA	Yes	12
5	Yes – 8 WOA	No	12
6	Yes – 8 WOA	Yes	12
7	No	No	12
8	No	Yes	12

NEWCASTLE DISEASE OUTREACH IN RISK FLOCKS IN SOUTHERN CALIFORNIA

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SUMMARY

Several cases of virulent Newcastle disease (vND) have been diagnosed in specific counties of southern California. While the outbreak has spread, an outreach team of members from the California Department of Food & Agriculture (CDFA) and the School of Veterinary Medicine at UC Davis has been focusing on making educational resources available to individuals with flocks at risk. A thorough understanding of these communities will not only inform us of effective distribution strategies of materials but will also establish a trusting relationship to help maintain animal health in these flocks. Conducting focus groups for these populations is crucial for collaborative relationships and consistent communication networks, which would permit the dissemination of information and avian health in the future.

INTRODUCTION

In May 2018, numerous cases of vND were confirmed in backyard, exhibition chickens of San Bernardino County in California, sparking an outbreak which spread to Los Angeles, Riverside, and San Diego counties. Overall, the outbreak has resulted in the euthanasia of over one million birds in backyard and commercial premises alike as well as in feed stores (2). Though a regional quarantine continues to be in place to prevent further spread of vND, containment of the disease has proven difficult due to the concentrated backyard bird numbers in suburban and urban areas (4). Unfortunately, while the number of positive cases began to decrease, violations of the regional quarantine in which individuals transported infected birds or contaminated equipment caused the resurgence of new vND positive cases (2).

Newcastle disease is known to be one of the most contagious diseases in poultry, with vND cases requiring reporting to the Office of International Epizootes (OIE) (3). Virulent Newcastle disease is a fatal viral disease which affects the respiratory, nervous, and digestive systems of poultry, with clinical signs such as coughing, nasal discharge, gasping for air, green watery diarrhea, neck twisting, and sudden death (1). The virus can cause an almost 100 percent death rate in unvaccinated poultry flocks and even kill vaccinated birds, potentially inducing overwhelming effects on the commercial poultry industry were it to spread to additional commercial facilities in the affected areas (1).

Interestingly, the areas currently affected by vND appear to be the same areas impacted by the 2002-2003 vND outbreak in backyard and commercial poultry in San Diego, Riverside, Los Angeles, and San Bernardino counties in California (2), indicating that individuals with backyard poultry still may not have the knowledge or the resources to prevent disease in their flocks. For this reason, educational outreach in this area is a vital and preventative tool to promote animal health and even aid in the eventual eradication of vND in southern California.

MATERIALS AND METHODS

Beginning in May 2019, a collaborative effort between CDFA, the UC Davis School of Veterinary Medicine, and the Western University of Health Sciences made possible monthly workshops held at the latter location, giving backyard poultry enthusiasts the opportunity to learn. At these workshops, representatives from each institution presented on topics like poultry diseases, biosecurity, vaccination, disease prevention, and updates on the vND outbreak. While attendee feedback was generally positive, unfortunately, workshops eventually garnered low attendance and outreach efforts were thus directed towards flocks at risk.

Such communities are often very closed groups with a strong sense of loyalty between them and a distrust toward official authorities (5). Considering the rapid spread of vND however, communicating with and establishing strong trust-based relationships with these groups is crucial. Thus, through networking, we conducted small focus groups to obtain a better insight

into these at-risk groups and to create continuous communication networks. The information obtained from the small flock owners in these groups helped us understand their community needs, the methods of communication they were likely to receive and check frequently, as well as the most suitable format to disseminate educational resources.

RESULTS

Their input has also allowed us to conduct organizational meetings with website developers, animal scientists, and extension officers to construct a webpage specifically for providing poultry resources. Through the communication networks established with these small flock owners at risk, we have had the opportunity to hold educational workshops in several locations throughout the affected counties with experts in pathology, poultry, and extension (Fig. 1) as well as hold informative radio interviews to reach broader audiences.

CONCLUSION

By providing small flock owners at risk with the educational resources they request and need, we can spread awareness on the severity of vND as well as knowledge on how to maintain flock health. Continuing these collaborative outreach efforts is crucial to maintain strong, trusting relationships with affected communities. Doing so would allow us to not only help reduce vND dissemination but also promote animal health through effective means of knowledge distribution for many years to come.

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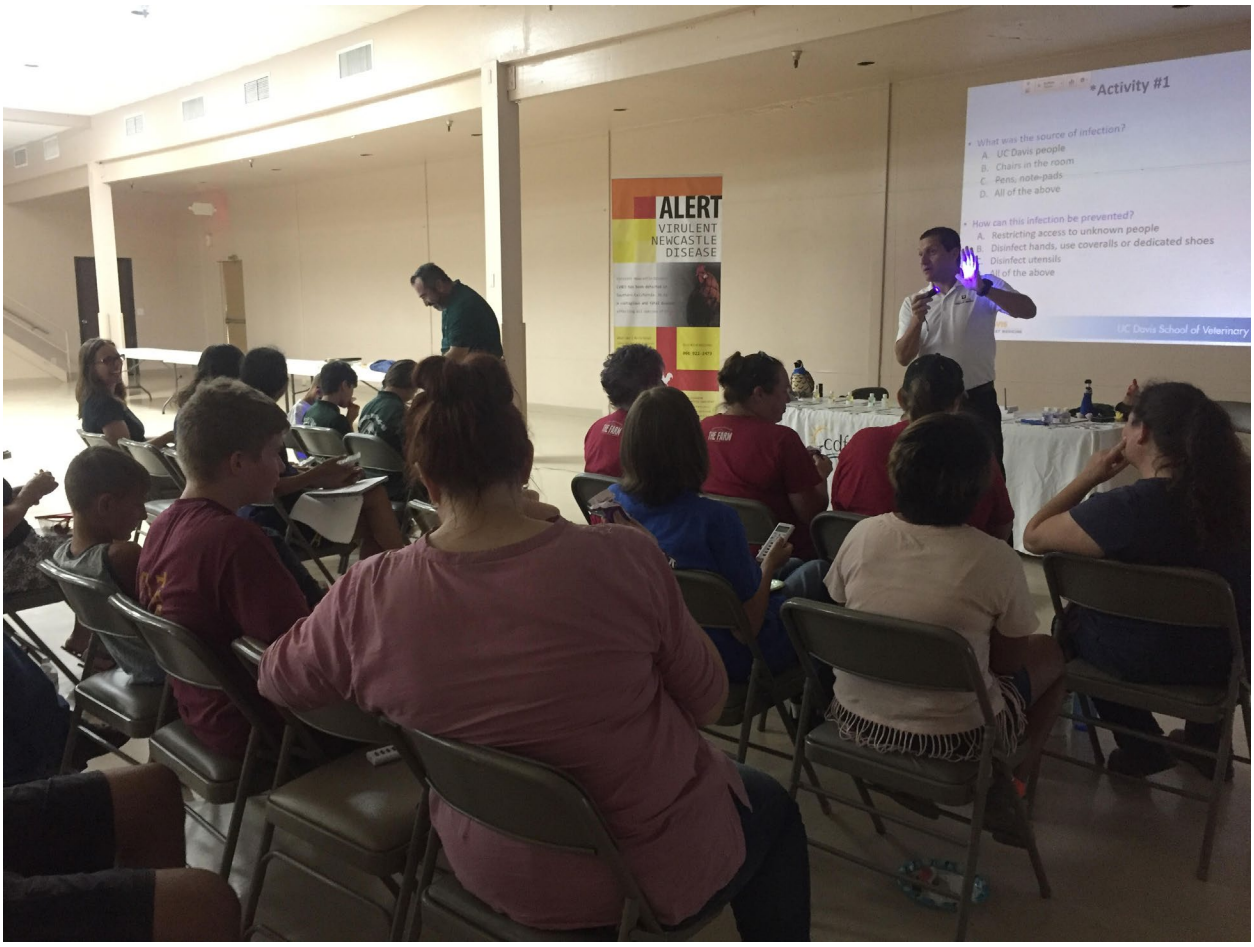
Figure 1. Members from CDFA and the UC Davis School of Veterinary Medicine teach about the importance of biosecurity at a community meeting in one of the counties affected by vND.



COCCIDIOSIS: OLD ENEMY WITH NEW TRICKS

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SUMMARY

Coccidia are generally present in almost all poultry-producing facilities and may cause a significant threat to the health of the birds based on the types and numbers of coccidia. The most prevalent species in commercial broiler houses are *E. acervulina*, *E. maxima*, and *E. tenella*. Species such as *E. hagani* and *E. praecox* are often found in animals usually on a synthetic anticoccidial. The frequency of having *E. maxima* in submitted fecal samples were approximately 80% irrespective of biological or pharmaceutical control programs. The control of coccidiosis in the population of chickens is essential; this disease negatively impacts the production potential of the affected animal. The two common methods of coccidiosis control are immunity via the host immune system or with the aid of pharmaceutics to reduce the number of coccidia being developed in the intestines of the chickens. A third program is using a combination of the two programs; we are still in a quandary as to the true value of this approach; time will be our best judge for this current practice.

INTRODUCTION

More than 70 years ago the enteric disease "coccidiosis" was one of great concerns for the poultry producers. However, with the discoveries and approvals of highly efficacious products (chemicals and ionophores) the industry had a period of reprieve from these agents. More recently, current trends are to reduce or remove many of the anticoccidial drugs. To add more challenge to already a complex matter, researchers have reported on coccidia that are aberrant in their patterns; these aberrant behaviors have led to the descriptions of cryptic and or variant species of Eimeria. The re-using the same litter for several flocks and shorter down time between flocks; these add a bit more complexity to the challenge. Other factors are birds per unit area and the inclusion of vaccines for the control of coccidiosis. However, many times vaccines are used in a bio-shuttle manner, where a vaccine is used at day of hatch and a prophylactic agent is added to the program at 10-12 days of age. Could the drug use be suppressing immunity within the bird population?

CONTROL

Drugs. The list of available anticoccidial that are routinely used in commercial broilers has gotten to be just a handful; this is due to the reduction in the efficacy of the product or regulatory or product choices and or combination of these issues. Practices of hoping to reduce or delay the onset of anticoccidial resistance via the shuttling of several anticoccidials in a rotation or within the life of the chickens within a flock. The vaccination of birds with drug sensitive strains of coccidia has shown great promise; it also enables the replication of drug sensitive organisms to become a residential population. These drug sensitive organisms have shown to enhance the efficacy of the anticoccidials (6).

Immunity. Vaccines can be applied before the chicks are hatched or post-hatched, to the embryo or the chicken, respectively. There are several types of coccidia vaccines; killed product is applied via injection as in the case of Coxabic® or ingested as in the case of Supracox®. There are two types of live coccidia vaccines; non-attenuated (Coccivac®, Immucox®, Advent® and Inovocox™) and attenuated (Paracox®, Livacox®, Eimeriavac®, Hipracox® and HatchPac®). The coccidia strains in most vaccines are drug sensitive (personal data) and therefore using a vaccine in conjunction with a drug may also be allowing the drug tolerant/resistant field strains to proliferate rather than allowing the replication of the drug sensitive population. A drug program may allow the animals to develop some level of immunity; however, this immunity may not be fully protective against a modest challenge. The shedders/carriers play a major role in the spread of the infectious agents (personal data). However, vaccinated animal that have developed protective immunity; the level parasitemia is low to none but the body is kept alert and in a defensive mode.

How do we assess what is happening? Monitoring coccidiosis is essential in achieving good control, maintaining good bird health and welfare and animal productivity. Commercially, these are achieved via several methods:

1) Routine necropsy – sub-population of birds are taken from multiple farms and multiple ages and intestinal health is evaluated.

2) Anticoccidial sensitivity tests (AST) - coccidia are harvested from multiple farms and multiple ages, characterized, enumerated then exposed to a battery of anticoccidial drugs in an in-vivo model.

3) Oocysts counts (OPG), evaluation of fecal droppings from several farms and several ages and enumerated based on types and numbers of coccidia.

4) Molecular assessments of the fecal excrement from farms have shown positive correlation with qualitative and quantitative results (3).

Each method has positives and negatives, but a combination of methods will provide useful and reliable information.

The importance of variant and or drug resistance and challenges. There is evidence of loss/reduced efficacy of numerous anticoccidial drugs to chicken and turkey coccidia. Most Eimeria species are relatively good antigens and may protect against different strains within species but offer no protection against other species of Eimeria. A few reports of poor protection and or variability in cross protection among strains of *E. maxima* (2, 4, 5). These reports have documented the uniqueness of these organisms but there were no comments on their prevalence in the industry. With malaria (*P. falciparum*) the wide spread use of antimalarial drugs has led to resistance (8). Also, symptomatic infections with high parasite loads are very likely the sources of resistant strains of *P. falciparum* (10).

With coccidia, the industry's recent practice of adding prophylactic agents following the application of drug sensitive vaccines may be defeating the initial intent of the vaccination process. Adding the agent during the period in which the animals should be auto-inoculating themselves with the drug sensitive coccidia may be increasing the drug tolerant population rather than the sensitive group. With this current practice, the endpoint is not known. Will this lead to new "cryptic" organisms, will our current programs (pharmaceuticals and or biologicals) be able to control them? Ramiro et al 2015, working with murine malaria species (*P. berghei* and *P. yoelii*) demonstrated that hybridization can occur between species; however, with a low frequency and the viability of the offspring was not determined. Blake et al 2015, showed the sharing of genetic information between two distinct strains of *E. tenella* yielding hybrid organisms. They speculated these genotypes may be capable of escaping drugs and or vaccination.

DISCUSSION

Malaria is a difficult disease to control, due to the high variabilities with the mosquitoes and protozoal parasites involved. Malaria is critical for younger patients, globally there are 219 million new cases per year with mortalities in excess of 0.44 million per annum. Effective tools and programs have been developed to combat malaria; however, the dynamics of hosts, parasites and environments interplay over time the parasites and mosquitoes have circumvented the tools and programs if not correctly used. Likewise, coccidia which are ubiquitous with commercial animal production and with the re-use of litter and extensive applications of pharmaceutics we may find our industry paralleling the malaria disease. Considering what has been seen with malaria and hybridization between two species also the sharing of genetic information within distinct strains of *E. tenella*; these combinations leading to hybrid organisms. However, the frequencies of occurrence of these organisms are relatively low. In our industry to achieve sustainable control over coccidia, researchers, company veterinarians and production personnel need to work jointly using all possible approaches and tools and constantly assessing for the best workable programs.

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INFECTIOUS BRONCHITIS VIRUS VARIABILITY, EXAMPLES IN NORTH AMERICA

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SUMMARY

Infectious bronchitis is a disease of the upper respiratory tract of chickens caused by a Gamma-Coronavirus (IBV). Severe economic losses are caused by IBV due to a reduction of egg production and/or its quality in layers in addition to poor feed conversion and increased condemnations in broiler chickens. The extreme variability of this virus is in part due to its RNA genome, which predisposes it to mutations and generates genetic variation. In addition, recombination events add to the variability of this virus. IBV variability was first described in 1956 by Jungherr since then, there have been dozens of serotypes and hundreds of genotypes reported. Variant IBV strains are those that, despite not being fully different from conventional strains affecting a geographic region, can escape from the immunity generated by conventional strains. At the genomic level, these differences can be greater or equal to 5% of the hypervariable region of the S1 gene. These variant strains are usually restricted to geographic regions and most of the time are transient, reason why diagnostics and epidemiological surveillance are crucial to determine their existence and persistence. This strategy helps planning preventative measures in the field.

INFECTIOUS BRONCHITIS VIRUS

IBV is a single stranded positive sense RNA virus of the *Coronaviridae* family, genus *Gammacoronavirus*. The viral genome comprises two untranslated regions (UTRs) in its 5' and 3' ends, two overlapping reading frames (ORFs) encoding the structural polyproteins 1a and 1ab, and the region encoding the main structural proteins i.e. spike (S), envelope (E), membrane (M) and nucleocapsid (N). Finally, two accessory genes ORF 3 and ORF 5 encode for proteins 3a, 3b and 5a and 5b (1). The S protein is located in the surface of the virus external membrane, during binding to the host cell it is post-translationally cleaved into the amino-terminal S1 (~535 amino acids) and the carboxy-terminal S2 (~627 amino acids) on a cleavage site rich in basic bases (2). The S1 portion of the S protein of IBV is responsible for viral attachment to host cells, virus variability and elicits neutralizing antibodies in chickens (3-6). S1 displays the most genetic and phenotypic variability among different IBV strains (3-5) and is therefore the best target to assess variability.

Variant IBV strains are those that, despite not being fully genetically different from conventional strains affecting a geographic region, are phenotypically different and escape from the immunity generated by conventional serotype-specific vaccines. At the genomic level, these differences can be greater or equal to 5% at the hypervariable region of the S1 gene. These variant strains are usually restricted to geographic regions and most of the time are transient (7), reason why diagnostics and epidemiological surveillance are crucial to determine their existence and persistence.

GENERATION OF IBV VARIANTS

Viral evolution. Viral evolution depends on two separated and independent mechanisms described by Mayr (8): (a) Generation of diversity, in which genetic/phenotypic variants are generated and serve as material for selection and (b) selection, in which the virions generated after the replication process are released in the environment. The survivors of that process will serve as the genetic pool for subsequent generations (9). IBV variability is generated by insertions, deletions and point mutations in addition to recombination events (10). IBV selection has been proved by Toro and Jackwood in 2008 (11, 12). The rapid evolution capability is what makes IBV highly successful in the environment and is the reason why IBV continues to spread and circumvent vaccination programs used in the poultry industry (13). Nowadays, we recognize the existence of dozens of serotypes and even more genotypes and variants. Variants have been recognized all over the world: Latin America, associated with kidney lesions (14); Africa, associated with swollen head syndrome (15); Egypt, associated with enteric problems (16). In Asia, variants have been present since at least 1979 (17). In Australia, where evolution has been independent from other countries in the world (18), variant IBVs have been isolated since 1960 (19).

Exotic genotype introduction. The introduction of exotic genotypes into a geographic region or

country can increase the local genetic pool of IBVs. This situation will increase chances of genetic recombination with local IBV strains and potentially generate IBV variants. The introduction of these IBV strains can be accidental, via contaminated poultry products and lack of biosecurity, and/or premeditated. The most common premeditated introduction is caused when vaccines from exotic genotypes are introduced as a tool for controlling IBV outbreaks caused by variant strains. There are several examples of generation of variant IBV strains after the premeditated introduction of exotic genotypes. One example is the event reported by Lee and Jackwood, where they describe the generation of the IBV genotype GA98 after the introduction and use of the DE072 vaccine. The IBV GA98 was the causal agent of extensive and costly outbreaks of infectious bronchitis in broilers in the State of Georgia (20). It is important to understand that, even though recombination events are not as common and effective for viral evolution as point mutations, insertions and deletions, they occur and are an important factor in IBV variant generation.

Vaccines and their use. A vaccine capable of inducing cross protection against different genotypes of IBV is of paramount economic and practical importance (21). Some commercially available vaccines are heterogenous, meaning that the predominant subpopulations in the vaccine are diverse and do not induce protective immune responses in chickens (22). The best example of these heterogeneous vaccines is the ArkDPI vaccine. Heterologous vaccines will either provide no protection or, at best, partial protection. If partial protection is achieved, it will ameliorate clinical signs but will not reduce shedding of challenge/field virus. High loads of challenge IBV strains combined with vaccine viruses creates the perfect scenario for variant generation. In summary, as it has been reported in the literature and anecdotally, live attenuated vaccine usage has a major role in the genetic profile of IB strains isolated in the field (23). Other than vaccine selection, vaccine application is crucial to avoid variant IBV generation. Currently available live IBV attenuated vaccines are mostly applied in the hatchery at day of age and by spray or drinking water between 10 to 15 days of age in broilers and at least 3 to 4 times in layers before laying onset. Massive application strategies for IBV vaccination are partially inefficient and usually result in vaccination failure (24). All these problems are aggravated if half or quarter doses are applied.

Population immune status. Population immune status plays a crucial role in the evolution of IBV, not only because of the effect of vaccination in selective pressure but also immunodepression that will allow the virus to evolve freely in the affected population. Numerous epidemiological studies using conventional and molecular virology techniques have demonstrated the capabilities of IBV to circumvent extensive vaccination programs which have been implemented since 1950 (13). Immunocompetent chickens show normal immune responses that are able to limit viral replication and limit generation of genetic variants for selection. In chickens showing less than optimal immune responses, the vaccine viruses are able to replicate in more individuals and viral populations different from the challenge strain become predominant (25, 26). This phenomenon can particularly happen when chickens are infected by highly prevalent and ubiquitous immunosuppressant viruses, i.e. chicken anemia (CAV) and infectious bursal disease virus (IBDV).

DETECTION, SURVEILLANCE, CONTROL, AND PREVENTION

Detection and surveillance of IBV. Accurate diagnosis and targeted surveillance is crucial to adequately prevent and control IBV variant rise and its detrimental effects (7). Other than orienting in the vaccine selection and vaccination strategy, accurate diagnosis will drive efforts to impede variant formation. IBV diagnostics are based on: (1) detection of the virus by molecular methods, i.e. reverse transcriptase polymerase chain reaction (RT-PCR) or RT quantitative PCR (RT-qPCR), targeting conserved genes such as M or N (9, 27) and (2) conventional methods such as virus isolation, serology and virus neutralization (28). The most used surveillance technique nowadays is the RT-PCR of a HVR of the S1 gene and sequencing of the amplicon with or without prior virus isolation (9). The size of the S1 fragment amplified by RT-PCR for surveillance is of major importance. While using the full S1 is the best choice because it allows the analysis of all three HVRs, it is less sensitive than amplifying a shorter fragment of S1 and requires a considerable amount of well-preserved virus RNA in the sample. On the other hand, amplification of small segments (between 300 and 500 bp) increases the test sensitivity, but short sequences overlook variations in other portions of the HVRs or recombination sites (29). While not as accurate as the full S1 gene, a segment of 750 nucleotides has been adequate for surveillance and evolution studies of IBV (9, 26). Surveillance data should be correlated with clinical signs, pathology, management, field data and vaccination records. If the viruses are highly pathogenic or cause severe productive losses, vaccine protection studies are recommended. If the protection provided by the available vaccines is not adequate, studies to assess

variability of the detected variant should be performed to determine if formulating a homologous vaccine to prevent outbreaks is safe (7). Sometimes in IBV surveillance, IBV variants not clearly associated with disease are detected. In this case, it is recommended to continue with close epidemiological surveillance. Generation of databases for specific regions is desired, since they allow for better interpretation of results (7).

Control of immunosuppressive viral diseases. Immunosuppression in poultry can be caused by several factors including stress, nutritional deficiencies, mycotoxins and viral diseases (30). The main viral immunosuppressive diseases reportedly linked with IBV cases in the field are IBDV and CAV. IBV cases in broiler chickens usually occur between 35 and 49 days of age and are potentially associated with immune deficiencies (7, 31). Even though it has not been reported as a direct cause of increase in IBV cases, Marek's disease virus (MDV) can cause immunosuppression affecting both humoral and cellular immune responses (32). This immunosuppression cause is very complex, poorly understood and in many cases under diagnosed. MDV vaccination protects against some of the aspects of immunosuppression but certainly not all (32).

Management. Proper management is a key component of a disease-free flock. Chickens can be exposed to stressors and infectious diseases. Any stress caused by poor management can affect the immune system and the consequences can be linked to upper respiratory tract infections (30). An example of poor management is poor ventilation. If intensive productive units are poorly ventilated there is a higher incidence of respiratory diseases. Increased particulate matter and ammonia in the environment (33, 34) in addition to the increased concentration of Gram-negative bacteria in the environment (35) cause irritation, inflammation and changes in the microbiota, inducing complex upper respiratory diseases (36). Understanding the presence and pathogenesis of risk factors is essential to a successful management for optimal health and welfare. At the same time, genetics and nutrition need to be recognized on their contribution to efficient production (30). Food-borne mycotoxins and suboptimal nutrition can diminish immune responses, particularly the innate reaction to pathogens (30). Biosecurity is the first barrier for pathogen introduction to poultry flocks. Lack of biosecurity or flaws will facilitate the introduction of IBV or any of the pathogens that cause immunosuppression (CAV, IBDV, MDV, reoviruses and adenoviruses).

In conclusion, IBV is highly variable and variants arise because of the constant evolution of this virus. There are factors that predispose the virus to more variability, e.g. introduction of exotic genotypes, poor vaccine selection and application and immunosuppressive diseases. Diagnosis, detection and surveillance are necessary to understand the circulating IBV genotypes. This understanding depends on an appropriate interpretation of results and helps generating a good preventative strategy and vaccination decisions. In addition, good practices, management and adequate control of immunosuppressive diseases help preventing the arise of variant strains. Prevention of endemic diseases in poultry is based on properly using the available tests for diagnostics and surveillance and interpreting results obtained with them. When these tools are not properly used, misleading information is shared and deceptive epidemiological data is implicated, which can affect future preventative strategies and predictions on IBV evolution.

(A full length article was submitted for publication in the Austral Journal of Veterinary Sciences.)

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DEVELOPMENT OF A NECROTIC ENTERITIS (NE) ANIMAL MODEL IN COMMERCIAL BROILER CHICKENS TO STUDY THE PATHOGENESIS OF NE AND TO EVALUATE VACCINE ANTIGENS AGAINST *CLOSTRIDIUM PERFRINGENS*

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ABSTRACT

Incidences of necrotic enteritis (NE) caused by *Clostridium perfringens* (*C. perfringens*) has increased in recent years due to the reduction of the prophylactic use of antimicrobials in the broiler chicken industry in Canada. NE as a secondary disease in broiler chickens has also been demonstrated due to immunosuppression associated with variant infectious bursal disease virus (vIBDV) infection. The objective of this study was to develop an animal model of NE in commercial broiler chickens.

Groups of broiler chickens were exposed to *C. perfringens* in feed or both *C*. perfringens in feed plus vIBDV at 18 days of age, along with an abrupt increase in dietary protein. A field isolate of *C. perfringens* containing α-toxin, netB, cpb2, and TpeL was used in this study. Four groups of broiler chickens (n=40/group) were used: group 1 = *C. perfringen*s in feed at days 20-23 of age, group 2 = vIBDV-SK09 at day 17 of age plus *C. perfringens* in feed at days 20-23 of age, group 3 = oral administration of *C. perfringens* (x109 CFU/bird) twice daily at days 20-23 of age and group 4 = no challenge control.

No mortality was observed in the control group however; mortality was observed at three days post-challenge in the groups challenged with *C. perfringens*. Total mortality in groups 1, 2 and 3 were 13.8%, 6.67% and 3.0% respectively. Gross pathological lesions typical of NE were observed throughout the small intestine. Histopathological lesions were seen in 100% of birds exposed to *C. perfringens* in feed, while 50% of birds had histopathological lesions in the group orally exposed to *C. perfringens*. It was demonstrated that the pattern of mortality, gross and histopathological lesions of this NE animal model were consistent with field cases of NE.

INTRODUCTION

NE is an important reemerging intestinal disease of broiler chickens caused by *C. perfringens*. *C. perfringens* is a gram positive, rod shaped, spore forming, anaerobic bacterium. *C. perfringens* affects broiler chickens between two to six weeks of age (1). Mortality can reach at 1% per day with a total mortality of 10-40% (3). Clinical signs such as depression, diarrhea, dehydration, ruffled feathers, low body weight, and increased feed conversion ratio (FCR) are typical with NE (2). It was estimated that the global economic impact associated with NE accounts for $6 billion/year in the broiler chicken industry (4). Economic losses will continue to rise since many countries are withdrawing the prophylactic use of antibiotics against NE. No effective commercial vaccine against NE is available to-date, therefore there is an urgent need to develop. The objective of this study was to develop an animal model of NE in commercial broiler chickens.

MATERIALS AND METHODS

Day-old Ross broiler chickens (n=160) were randomly assigned into four experimental groups each consisting of 40 birds/group (group 1= feed administration of *C. perfringens*; group 2 = vIBDV-SK09 + feed administration of *C. perfringens*; group 3 = vIBDV-SK09 + oral administration of *C. perfringens*; and group 4 = saline control). vIBDV-SK09 was administered orally (1x103 EID50/bird) at 17 days of age to predispose birds to NE. Broiler chickens were fed antibiotic free chicken starter with 20% protein for 18 days followed by an abrupt increase to 28% protein. Classical clinical signs associated with NE such as depression, anorexia, ruffled feathers, brown foamy diarrhea and deceased mobility were observed in all *C. perfringens* exposed groups.

The challenge strain of *C. perfringens* had α-toxin, netB, cpb2, and TpeL toxin genes. *C. perfringens* was grown in cooked meat medium (Sigma Aldrich) for 24 h at 37 °C under anaerobic conditions. 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 1×109 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*. *C. perfringens* was administered twice daily either in feed or orally for three consecutive days. Birds were observed for clinical signs and mortality post-challenge, and the experiment terminated after five days' post-challenge. Intestinal track of all the birds were examined for macroscopic lesions of NE and histopathological lesions.

RESULTS AND DISCUSSION

Classical clinical signs associated with NE such as depression, anorexia, ruffled feathers, brown foamy diarrhea and deceased mobility were observed in all *C. perfringens* challenge groups. Broiler chickens in group 4, not exposed to *C. perfringens*, did not exhibit any clinical signs. Relatively severe clinical signs were observed in groups 1 and 2 (in feed groups) compared to group 3 (oral challenge). No mortality was observed in the control group while mortality was observed at three days' post-challenge in groups exposed to *C. perfringens*. Total mortality in groups 1, 2 and 3 were 13.8%, 6.67% and 3.0% respectively. Gross pathological lesions typical of NE were observed throughout the small intestine. Histopathological lesions were seen in 100% of birds exposed to *C. perfringens* in feed, while in the group orally exposed, 50% had histopathological lesions. Birds exposed to *C. perfringens* had classical histopathological lesions such as severe diffuse necrosis of villi associated with bacterial colonization with infiltration of minimal inflammatory cells.

We have repeated this NE animal model and found that it is a reproducible animal model in commercial broiler chickens in a laboratory setting. It was demonstrated that the pattern of mortality, clinical signs, gross and histopathological lesions were consistent with field cases of NE. We are planning to use this animal model in our ongoing work of developing a vaccine against NE and studies exploring pathogenesis of *C. perfringens* in commercial broiler chickens.

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INFLUENCE OF LITTER CONDITIONS ON SPORULATION AND SURVIVAL OF COCCIDIA OOCYSTS

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ABSTRACT

The life cycle of *Eimeria* spp., commonly referred to as "coccidia," contains oocysts as the external stage. The oocysts need to sporulate in the environment, i.e. they need to form sporocysts and sporozoites, which infect the host after oral uptake and destruction of the outer wall in the upper gastrointestinal tract. The oocysts are very resilient in the environment, which contributes to their nearly universal occurrence in chicken flocks. However, sporulation as well as survival of oocysts depend on the environmental conditions, i.e. managing environmental conditions like temperature, humidity and composition of the matrix in which the sporulation takes place is an opportunity to influence the infection pressure.

Temperature seems to be the factor with the most obvious influence on oocyst development. In fecal suspension, the temperature allowing the oocysts to sporulate in the shortest time was about 30°C (1,2). While this information is important for laboratory procedures, there is little relevance for poultry flocks, where oocysts are in litter, and temperature is set according to the needs of the chicks. However, heating houses and litter between flocks might destroy oocysts, but no information on this aspect is available.

Litter moisture is often regarded the important parameter, and also a parameter that is relatively easily controlled by ventilation. However, sporulation rates were not significantly different between litter with a relative humidity of 40 versus 80% (3). Only very dry litter with a relative humidity of 16% allowed for a higher sporulation rate compared to litter with a relative humidity of 62% (4). Sporulation rates are higher in litter than in pure feces, presumably due to higher concentrations of ammonia (3). The addition of various litter treatments like superphosphate, meta-bisulfide, and charcoal reduced oocyst counts (5), while covering litter with plastic had no effect (6).

In litter kept under conditions similar to those being found in chicken houses, oocysts begin to disintegrate as soon as one day after being shed, but viable oocysts were detected as long as 50 days later (7). There are also indications that the behavior of the different *Eimeria* spp. might differ, partially explaining why some species are more prevalent than others (8).

The aim of the present investigation was to establish a laboratory model that can be used to systematically test the influence of litter treatments between flocks on sporulation and disintegration of *Eimeria* oocysts.

Two pilot experiments were conducted testing the influence of temperature and humidity on oocyst recovery, sporulation, size, and oocyst disintegration. The first experiment investigated the influence of temperature and humidity on oocyst counts and sporulation, while the second experiment focused on oocyst size and disintegration and included replicates to estimate repeatability.

In the first experiment, 200 g of dry litter and 200 g feces containing *Eimeria maxima* oocysts were mixed and divided into four aliquots, each stored in a plastic box, covered loosely with a lid. To two aliquots, 20 ml of tap water were added. One dry aliquot and one aliquot to which water had been added were stored 20 cm under a heat lamp; the other two aliquots were stored at room temperature. Every four days until the end of the experiment after 28 days, samples were collected and processed with routine procedures for counting in a McMaster chamber. When counting the oocysts, the percentage of sporulated oocysts was determined.

The two aliquots kept at cooler temperature had the higher oocyst counts. The effect of the added water differed. At the cooler temperature, oocyst counts tended to be higher in the dryer aliquot, while at the warmer temperature the reverse effect was observed. The percentage of sporulated oocyst was constantly higher in the aliquots kept at the cooler temperature and reached 50% at the end of the experiment. In contrast, in the aliquots stored under the heat lamp, only on one day more than 10% of the counted oocysts were sporulated. There were no systematic differences between the aliquots with and without added water.

In the second experiment, 400 g of dry litter and 400 g feces containing *E. acervulina* oocysts were mixed and divided into eight aliquots, each stored in a plastic box, covered loosely with a lid. To four aliquots, 25 ml of tap water were added. All boxes were weighed. Every three days until the end of the

experiment after 18 days, boxes were weighed again and water was added to the original weight to compensate for evaporation of water. On the same days, samples were collected and processed with routine procedures for counting in a McMaster chamber. In addition, photos of 50 different areas of the chamber were taken. In these photos, length and the width of 100 oocysts on days 1 – 10 and of 20 oocyst on days 13 – 22 were measured, and the size of the oocysts was calculated. Measured oocysts were classified as intact or damaged. Oocyst counts and sizes as well as the percentage of damaged oocysts were tested for significant differences between treatments ($P < 0.05$) by t-test using R 3.6.0 (9). Oocyst sizes were tested for differences between damaged and un-damaged oocysts by t-test using R.

The average oocyst counts were higher in the wet litter during the first ten days. Thirteen days after the start of the experiment, oocyst counts in the dry group were higher, later differences were not significant. In contrast, the percentage of damaged oocysts did not differ during the first ten days, while later the percentage of damaged oocysts was higher in the dry group. Oocysts were on most days larger in the wet group. Damaged oocysts were in average smaller than intact oocysts.

The first experiment indicated that coccidia oocysts disintegrate slower in a cooler environment, while at the same time sporulating at a higher rate. For this reason, the second experiment was done only at cooler temperatures. In it, replicates were added to allow a statistical analysis, and oocyst and size and signs of damage were added as parameters. Sporulation status had to be dropped as a parameter, because the quality of the pictures did not allow a reliable assessment. The results showed oocysts counts were initially higher in wet litter. However, there was also a high variability and seemingly increasing oocyst counts over time in spite of thoroughly mixing feces and litter when setting up the experiment and every time samples were taken.

Considering the, in tendency, higher oocyst counts in wet litter, it was surprising that the proportion of damaged oocysts was higher in dry litter. One potential reason may be that damaged oocysts took a longer time to disintegrate completely after initial damage in dry litter. Damage was also indicated by a smaller size compared to oocysts that looked intact. However, there was a high variability in size and a large number of oocysts had to be measured, which is impractical for a high number of samples as envisaged in experiments to come. These experiments will explore the effect of litter treatments under cool and wet conditions. Oocyst counts, sporulation status and damage were shown to be suitable parameters.

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FEED THE FUTURE INNOVATION LAB FOR GENOMICS TO IMPROVE POULTRY: INCREASING FOOD SECURITY IN AFRICA BY ENHANCING RESISTANCE TO NEWCASTLE DISEASE AND HEAT STRESS IN CHICKENS

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SUMMARY

Homestead and small-scale poultry production has tremendous potential for alleviation of malnutrition and poverty in climate-stressed rural communities in Africa. Poor animal health and husbandry practices limit village poultry production. Newcastle disease (ND) is the number one constraint of raising poultry in Africa, causing high mortality among village flocks. This research and development program seeks to improve poultry production by households and small farmers, and thereby improve food security, nutrition, and livelihoods in Africa -- key goals of the USAID Feed the Future Program. Genetics plays a significant role in resistance of poultry to NDV infection.

Our program is applying advanced genetics and genomics to sustainably enhance innate resistance to ND and heat stress in chickens adapted to African environment. In the past six years, we identified the most robust genes and markers for genetic resistance through an integrated analysis of NDV challenge experiments and genomic analyses of well-characterized chicken lines under strictly controlled environmental conditions by RNA-seq and genome-wide association analysis (GWAS) using chicken 600K SNP panel, and of six African indigenous chicken ecotypes by GWAS.

Our results confirmed the polygenic control of resistance to NDV and suggested that viral load and antibody titer following infection are important parameters for evaluating disease resistance. We estimated heritabilities for these traits in African chicken to be moderate to high (0.14 – 0.55), which indicates that selection to improve these ecotypes for resistance to ND is feasible. In addition, natural exposure trials of about 3,000 birds of African ecotypes with velogenic NDV strains were conducted to identify associations of markers with viral shedding, survival time, and anti-NDV titers.

Based on our results, a low-density 5K SNP panel for genetic resistance to NDV was developed to select and breed local ecotypes with enhanced resistance to ND. In addition, we conducted a pilot study to evaluate natural antibodies as a biomarker associated with resilience to ND. Genetic heritability was 0.14 (medium). Natural antibody levels were positively correlated with viral clearance rate and negatively correlated with viral load at 6dpi in NDV La Sota trials. We are currently assessing correlations of disease resistance traits with crucial production traits, such as egg production and growth rate. We are also conducting value chain assessment and developing a business plan to enable distribution of improved chicken lines to rural farmers with a focus on women who would benefit the most of an increase in resistance and productivity.

The study was funded by the US Agency for International Development Feed the Future Innovation Lab for Genomics to Improve Poultry AID-OAA-A-13-00080 (H. Zhou).

OBTAINING SUCCESSFUL COCCIDIOSIS CONTROL IN TURKEY PRODUCTION THROUGH VACCINATION

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SUMMARY

According to surveys conducted recently by the Association of Veterinary Turkey Practioners in the United States (US), coccidiosis in turkeys has been identified as one of the top priorities for research and development in the US turkey industry. More specifically, they would like work done looking at the most effective non-ionophore/antibiotic strategies for controlling coccidiosis, and to determine best practices for using coccidial vaccines.

As a result of this industry interest and demand, a novel coccidiosis vaccine, Immucox® T, for turkeys was released in the United States in late 2018. Leading up to its release, and immediately thereafter, efforts were made to fine tune and maximize the effectiveness of this vaccine in the industry.

Successful coccidiosis vaccination requires that the vaccine be handled and stored appropriately, and that it be administered in a way that maximizes the uptake of the vaccine oocysts. This is especially true for turkey poults which typically undergo treatment processes in the hatchery that may affect vaccine uptake "fitness." In addition to ensuring a good initial vaccine oocyst uptake, several subsequent cycles must take place successfully in the field without interruption. This "re-cycling" is entirely dependent on management factors during the first 4-5 weeks of life in a turkey barn.

This paper will focus on experiences in the field related to this vaccine's use, as well as best management practices in both the hatchery and in the field, in order to optimize application, uptake, re-cycling, and the development of immunity in turkeys against coccidiosis.

INTRODUCTION

According to surveys conducted recently by the Association of Veterinary Turkey Practioners in the US, coccidiosis in turkeys has been identified as one of the top priorities for research and development. As a result, of this industry interest and demand in the US and Canada, a novel coccidiosis vaccine, Immucox T, for turkeys was developed and licensed in Canada in 2016. The product further received a conditional license and was authorized for release in the US in late 2018. Leading up to its release, and immediately thereafter, efforts were made to fine tune and maximize the effectiveness of this vaccine in the industry.

MATERIALS AND METHODS

Hatchery considerations. Proper vaccine handling & mixing should be performed. Live coccidia oocysts are date and cold chain sensitive, so must ensure oocysts remain viable prior to administration. Always check expiration date and maintain proper cold chain (36-46F) during storage and handling. Use of gel diluent via gel droplet administration in the hatchery to day of age poults is encouraged. Gel spray should be checked for appropriate quantity and for proper spray pattern in the poult tray and adjusted if necessary. Poults should be pulled at appropriate times, as poult fitness affects vaccine uptake. Providing extra light is helpful for enhancing preening of gel droplets. Tongues can be checked immediately after vaccination for gel uptake.

Brooder barn considerations. Allow birds to shed first oocysts in larger areas of the barn (minimum of $1ft^2$). Target bird release around 4d (before the first oocyst shedding) & again around 10d (before second shed period) if done in stages. Litter/air moisture must not become excessive. Oocysts need at least 25% litter moisture and air humidity of at least 35%. Avoid any "wet" litter (> 40%), especially around feed or drinker areas. If litter becomes wet, it should be removed and replaced with dry litter as soon as possible. Tilling or forking of litter can be performed as needed but may want to try to avoid oocyst shed and uptake periods.

RESULTS AND DISCUSSION

Managing through reactions. Bird behavior may change depending on vaccine cycle stage. This is normal and expected. Pacing, vocalization, huddling as if feverish, uneasiness, changes in fecal or cecal droppings, litter consumption, etc… are common clinical signs observed when poults are going through any form of enteric stress, including coccidiosis vaccine reactions. Supplemental interventions

(temperature adjustment, use of attractants to stimulate feed intake) may be necessary to help birds through cycle stress periods. Some sort of anti-inflammatory products (water soluble aspirin) may also help poults "feel better." The administration of anything with potential anticoccidial properties should only be performed under strict guidance of your veterinarian, as some products (i.e. essential oils, tetracyclines, sulphonamides) can interfere with proper vaccine oocyst recycling if used inappropriately. If coccidiosis related mortality occurs, intervention with low to moderate levels of amprolium or another anticoccidial agent should be considered for 1-2d. Vaccine oocysts are extremely sensitive to anticoccidials, so it shouldn't take much to make a big impact. Ideally, NO treatment should be needed. Also avoid extra stress during oocyst re-cycling (e.g. HE vaccination).

DEVELOPMENT OF A RAPID NANOPORE SEQUENCING ASSAY FOR DETECTION OF *MYCOPLASMA GALLISEPTICUM* AND *MYCOPLASMA SYNOVIAE* IN POULTRY

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SUMMARY

Rapid, accurate detection of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) is essential for implementation of treatment and control programs in poultry industries. Primer design and initial development of a targeted nanopore sequencing assay and bioinformatic workflow for simultaneous detection of MS and MG are described. Application of the multiplex targeted sequencing assay to pure cultures of MS WVU 1853 and MG Rlow strain resulted in over 99% pairwise identity to reference strains of MS and MG, except for one of three targets for MG. The assay was designed to be customizable by addition or omission of either DNA or RNA target sequences, meaning it would be useful in creation of a poultry respiratory panel to detect and type relevant bacterial and viral pathogens of industry importance.

INTRODUCTION

MS and MG are avian respiratory pathogens responsible for severe economic losses for the poultry industry worldwide. Often, respiratory diseases are complicated, multifactorial (e.g. coinfections of multiple agents), and may have similar clinical presentation. This can consequently result in redundant, expensive testing and extended turnaround times for conclusive diagnosis. Sensitive tests that allow very early detection of MS and MG are an important part of the control of these infections. As PCR protocols are amenable to high throughput testing, they have the potential to be used not only as confirmatory tests but also as screening tests for the early detection of avian pathogens. While realtime PCR has become the gold standard for rapid detection of pathogens, there are limitations to the assay, such as available fluorophore channels, that render sequencing techniques as preferable alternatives. Sequencing technologies can be altered and added to in order to allow even more samples to be processed at once for more streamlined and comprehensive diagnosis. For instance, targeted sequencing allows specific regions of interest to be amplified for better sequence detection and it has the potential for thousands of targets to be amplified in a single reaction tube (1). For this reason, we hypothesize that highly specific primers targeting genes of MS and MG can be utilized to develop a diagnostic multiplex sequencing scheme which will identify and type avian mycoplasmas in a more accurate and higher capacity than quantitative PCR or metagenomic sequencing techniques.

MATERIALS AND METHODS

Primer design. Forward and reverse primers were designed to target conserved genes of MS and MG using Primer3Plus and NCBI. Criteria for primer design included ensuring all primers designed had similar annealing temperatures and would amplify targets at least 700 bp in length. Designed primers were all unique to the organism targeted and do not form primer-dimers with each other. Primers for MS amplified portions of the tuf, nanA, ugpA, and ITS genes; primers for MG amplified portions of the mgc2, vlhA, and ITS genes.

Samples. Pure cultures of MS WVU 1853 and MG Rlow strains were grown to stationary phase and DNA extracted. MS+, MG+, and MS/MG negative tracheal swab preps submitted to PDRC Diagnostic Lab were collected and DNA extracted.

PCR. Targets for MS and MG were validated for single target amplification using the SuperScript III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase according to the manufacturer instructions. Primers were then pooled to develop a multiplex PCR for all targets and concentrations of primers adjusted in the pool to ensure all targets would be amplified. Agarose gel electrophoresis was used for initial verification of amplification.

Library Preparation and Nanopore Sequencing: The ONT PCR Barcoding Kit (Oxford Nanopore Technologies, SQK-PBK004) was used according to the manufacturer's instructions to prepare the sequencing libraries. Barcoded and pooled libraries were loaded onto the MinION (Oxford Nanopore Technologies) and sequencing was run with live basecalling.

Bioinformatic workflow. The MinKNOW program performed live basecalling while sequencing was performed. Basecalled reads were then run through Porechop to demultiplex the barcodes and trim the adapters. Geneious Prime is then used to sort sequences according to size, sequences within the size ranges of targets are assembled, and consensus sequences are identified using BLASTn. Pairwise identities of target regions to MS ATCC 25204 and MG Rlow strains were recorded.

RESULTS

Primer design and RT-PCR. All primers run in a single target RT-PCR amplified their intended targets based on the size of bands returned. The target for MG vlhA also returned a band of approximately 650 bp when run against MS WVU 1853. There was also an additional band for the MG vlhA target when run against MG Rlow strain. Targets were pooled for multiplex RT-PCRs of:

1.) All MS targets.
2.) All MG targets.
3.) All MS and MG targets.

For the MS multiplex, all four targets returned bands at the expected size. For the MG multiplex, both bands that were previously seen with the vlhA targets showed up, however it is difficult to see if mgc2 and ITS are both present due to size similarities of the targets. For the multiplex of all 7 MS and MG targets, gel analysis revealed a basepair size profile that would be expected when comparing to multiplexes of individual *Mycoplasma* species.

Nanopore Sequencing. For the MG multiplex, pairwise identity of MG Rlow culture amplicons to MG Rlow (AE015450) were as follows: MG vlhA (99.6%), MG mgc2 (99.3%), and MG ITS (97.5%). For the MS multiplex, pairwise identities of MS WVU 1853 culture amplicons to ATCC 25204 (CP011096) were as follows: MS tuf (99.7%), MS nanA (99.5%), MS ugpA (99.3%), and MS ITS (99.7%). Finally, for the MS/MG multiplex, pairwise identities to either MG Rlow or MS ATCC 25204 were as follows: MG vlhA (99.6%), MG mgc2 (99.7%), MG ITS (no result), MS tuf (99.8%), MS nanA (99.2%), MS ugpA (99.3%), and MS ITS (99.7%).

DISCUSSION

Selection of targets for the amplicon sequencing scheme was built upon targets currently used in diagnostic MLST assays for MS and MG (2-6). In order to implement this amplicon sequencing scheme in a diagnostic setting, adoption of quality control will be necessary. For instance, addition of primers targeting genes, such as collagen, present in all tracheal swabs would increase confidence that submitted samples are truly negative if no amplicons of pathogens are sequences.

When all seven targets were multiplexed, the MG ITS target did not amplify sufficiently to sequence successfully. A likely explanation for this would be that all other primer pairs in the multiplex amplified their intended targets far more efficiently than MG ITS primers and the reactions utilized free dNTPs before they could be used to amplify the MG ITS target. Should MG ITS continue to be included in the assay, adjustments will need to be made to the reaction mix to enable MG ITS to amplify. These adjustments may be made by altering primer concentrations, adding more dNTPs, or even redesigning the primers.

While MS and MG have DNA genomes, the decision was made to develop the amplification step as an RT-PCR. The assay was designed to be customizable by addition or omission of target sequences, meaning it would be useful in creation of a poultry respiratory panel to detect and type both bacterial (MS, MG) and viral pathogens of industry importance, such as infectious laryngotracheitis virus and Newcastle disease virus.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Marianne Dos Santos, Ruth Wooten, Rosetta Barber, Tristan Reed, and Mohammadreza Ehsan for their guidance and/or assistance with sample collection and processing. The authors also thank the U.S Poultry & Egg Association (USPEA) and the U.S. Department of Agriculture (USDA) for funding this study.

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BOVINE SERUM ALBUMIN (BSA) TO DESIGN MECHANICALLY, THERMALLY, AND CHEMICALLY STABLE NANOPARTICLES

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ABSTRACT

Recently, we have used bovine serum albumin (BSA) to design mechanically, thermally and chemically stable nanoparticles. BSA is nontoxic. BSA is a natural drug delivery vehicle that has the ability to bind a number of different small molecules, dyes, peptides, hormones, and drugs. Two antigenic peptides of spike protein (S) of IBV were covalently linked to the COOH groups on the FluoDot carriers purified, and then injected into chickens for immune-protection against avian bronchitis viral infection.

MONTANIDE™ ISA 71 R VG FOR LONG TERM PROTECTION VACCINE AGAINST INFECTIOUS CORYZA

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SUMMARY

Infectious coryza (IC) is an acute disease in chickens caused by *Haemophilus paragallinarum*, which affects the respiratory system. This widespread disease causes high economic losses due to heavy impact on laying production and increased culling rate in growing chickens. *Av. paragallinarum* has been classified into three serovars with many countries reporting the presence of all serovars. The development of trivalent inactivated IC vaccine is the better prophylactic strategy to protect chickens. However, adjuvants are required to improve inactivated vaccine efficacy. Montanide™ ISA 71 R VG (ISA 71R) is an adjuvant currently use in poultry industry. It is designed to resist destabilizing antigenic media such as bacterial antigens and allows flexible ratio of oil and antigenic media, needed for the formulation of multivalent vaccines.

In this study, we evaluated the performance of Montanide™ ISA 71 R VG formulated in a trivalent vaccine in a large scale IC trial.

In a first trial, 30 chickens per group were injected at D0 and D21 with IC trivalent vaccines either based on ISA 71R or standard adjuvant. Galenic properties of vaccine formulations were tested in laboratory conditions. Vaccine efficacy was assessed by a virulent challenge at D35. ISA 71R based vaccines were stable and induced 100% protection against all the valences of the infectious coryza vaccine.

For the large scale trial, approximately 100 000 chickens distributed in four farms located in two different states in South India, were vaccinated with the trivalent IC vaccine in a prime/boost protocol. For seven days after each vaccination, injection sites were inspected for swelling and mortality was monitored. No untoward effects at the injection site were observed after injection in each farm. The daily mortality number following the vaccination was either lower or similar to the standard mortality observed in a healthy flock. Moreover, egg production and mortality were also recorded weekly from beginning of laying cycle up to 17 months of age and compared with standard mortality and standard egg production. The egg production and the mortality rate of vaccinated flocks with ISA 71 R was also similar to what is observed in a healthy flock for each farm.

These results show that Montanide™ ISA 71 R VG is a safe adjuvant to be used in multivalent bacterial vaccines and protects chickens against bacterial disease such as IC in real field conditions.

INFECTIOUS LARYNGOTRACHEITIS CASES IN THE FRASER VALLEY OF BRITISH COLUMBIA 2017 TO 2019

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SUMMARY

Infectious laryngotracheitis (ILT) in commercial poultry has been endemic in the Fraser Valley of British Columbia for many years. With the success in the increased awareness on biosecurity, the implementation of effective vaccination programs, enhanced management practices and communication among the industry, the number of ILT cases reported has reduced significantly since 2009 (Figure 1). However, ILT has not been eradicated in the Fraser Valley. Sporadic cases have been reported from 2011 to 2017 and a recent spike in the number of cases over the last two years. The ILT cases were reported in majority poultry commodities including broiler breeders, broiler chickens, table-egg layers, specialty chickens, backyard flocks and game birds.

Infectious laryngotracheitis is an acute, highly contagious respiratory disease caused by an avian herpesvirus (Gallid Herpesvirus 1) (2, 3). There is no evidence of vertical transmission, but the virus can be transmitted horizontal from direct bird-to-bird contact (2). The virus can also be spread in fomites through humans, shared equipment, feed and water sources, and vermin (2, 3). Infected birds start to show clinical signs five to 12 days after natural exposure (2, 3). Birds that have recovered from an active infection or have been infected subclinically can become life-long carriers (2). Latent virus can be reactivated when the birds are stressed and/or immunosuppressed (2). The disease can persist for up to six weeks in the flock, and losses are associated with poor performance and mortality (3).

The infected flocks typically report a sudden spike in mortality and morbidity. In some cases, the first clinical sign is a reduction of feed and water consumption. Other clinical signs reported include conjunctivitis, blood-tinged nasal and ocular discharge, lethargy, head shaking, rasping, and change of vocalization. Mortality usually increases from three to seven days. Flocks that were vaccinated with the recombinant vaccines at the hatchery tend to report lower mortality and morbidity rate.

Diagnosis of ILT is confirmed with gross pathology, histology, and molecular diagnosis (PCR) findings. Postmortem examination showed various degree of tracheal lesions: tracheal congestion, 'muddy' appearance of the tracheal mucosa, mucopurulent plugs, and hemorrhagic tracheitis. Occasionally, birds also have mild to moderate conjunctivitis with ocular and nasal discharge. Majority of the birds submitted were emaciated and dehydrated. Almost all the cases submitted did not show signs of concurrent diseases. Histological finding is characterized by intranuclear inclusion bodies in the tracheal epithelium (2).

Like other viral diseases, there is no effective treatment for ILT. Effective prevention and control of ILT revolves around proper biosecurity, effective vaccination programs, and timely communication. Immediate self-quarantine and biocontainment are important to minimize further spread to nearby premises. Critical components include a double entry system to the barn, proper dead bird disposal and barn clean-out procedures that limit the spread of the virus. Manure are being heat treated prior to leaving the barn and being handled carefully to reduce dust. Supportive therapy through water, adjustment of environmental parameters and management practices are implemented to mitigate mortality and morbidity. Occasionally, broiler flocks are shipped earlier with planned route to limit the spread of virus and the loss in mortality. ILT is a provincially reportable disease which allows notification to be released to commercial industry to improve precautionary measures to limit the spread.

Recombinant ILT vaccines, chicken embryo origin (CEO) vaccine and tissue culture origin (TCO) vaccines are the three main types of attenuated live vaccines that are commercially available. CEO vaccines can revert to virulence and cause clinical disease and mortality in naïve flocks (1,3). Previous studies suggest that most outbreak strains in the Fraser Valley were closely related to the CEO vaccines. In 2011, local veterinarians made the decision to not to use CEO vaccines; however, the use of CEO vaccines is not regulated and there have been anecdote reports of the use CEO vaccines in the area. A current molecular epidemiologic study is underway to help determine the ILT strains that were involved in the recent cases.

This presentation will focus on the epidemiology, control/management, and prevention of ILT in the Fraser Valley of British Columbia.

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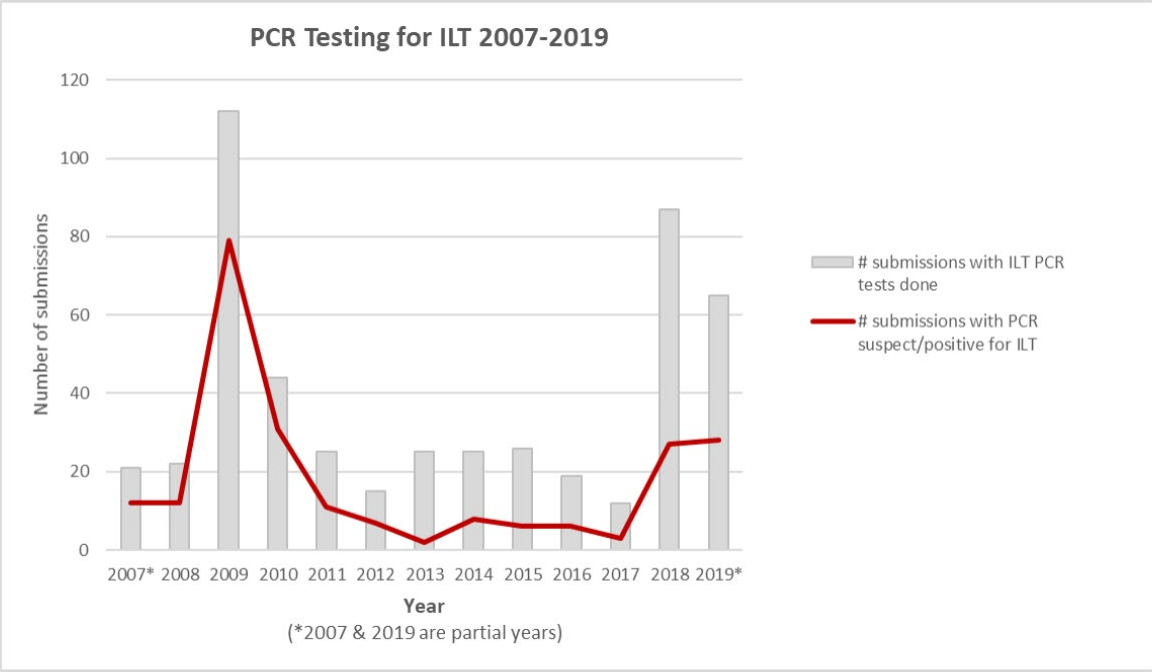
Figure 1. PCR testing and suspect/positive ILT results for submission from 2007-2019 in British Columbia (1).



INFECTIOUS CORYZA AND MYCOPLASMOSIS IN BACKYARD CHICKENS IN BRITISH COLUMBIA

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SUMMARY

Raising backyard chickens has become a very popular hobby in North America. The majority of the municipalities in British Columbia allow residents to keep backyard poultry. It is estimated that the Fraser Valley of British Columbia, has over 10,000 small flocks. The majority of the flocks range from five to 30 birds (Personal communication, T. Redford, Animal Health Center BC, 2020). While keeping poultry can be an enjoyable and rewarding experience, it is common to see disease and management problems in backyard poultry.

The control and prevention of infectious diseases in backyard poultry has represented a challenge due to various factors, such as frequent exchange and mixing of birds; inadequate biosecurity; the lack of regulations; limited access to specialized veterinary services; poor communication; misleading online information; and unregulated live markets. These factors increase the risk to the neighboring commercial poultry industry.

In recent years, infectious coryza and mycoplasmosis represent the two most prevalent infectious diseases in backyard chicken flocks in British Columbia. With the lack of database and regulations on small flock, it is difficult to estimate the prevalence of these diseases. It is estimated that approximately 30 to 50 percent of the small flock submissions are affected by one of mycoplasmosis or infectious coryza, with a small percentage affected by both (Personal communication, T. Redford, Animal Health Center BC, 2020). The presentation will focus on the infectious coryza and mycoplasmosis in backyard poultry flocks and the on-going challenge of the prevention and control of these diseases in small flocks.

ALTERNATIVE STRATEGIES AND MANAGEMENT OF COLIBACILLOSIS WITHOUT THE USE OF ANTIBIOTICS: CASE STUDIES FROM MULTIPLE COMMODITIES

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SUMMARY

In poultry diseases cases in the Fraser Valley of British Columbia, morbidity and mortality caused by *E. coli* have been associated with environmental, management, and/or concurrent disease challenges. In organic and raised-without-antibiotics programs, the management of E. *coli* infection relies heavily on strict discipline and attention to details relative to husbandry. These interventions include the identification and elimination of predisposing factors; the enhancement of immunity; competitive exclusion through the administration of live *E. coli* vaccine, acidifiers, and probiotics; and enhanced biodefense. This presentation will include four to five case studies involving *E. coli* infection management. The cases will cover all the following commodities in poultry: turkeys, layers, broiler breeders/broilers, and pigeons.

THE EVOLUTION OF CLINICAL SIGNS AND GROSS LESIONS ASSOCIATED WITH DMV1639 INFECTIOUS BRONCHITIS VIRUS FIELD INFECTIONS

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SUMMARY

The purpose of this presentation is to describe and show clinical signs and gross lesions associated with DMV1639 infectious bronchitis virus (IBV) in the field observed since 2015. In broilers, the signs and lesions have evolved from those associated with kidney damage to predominantly respiratory. Damage to the reproductive tract has also been documented. Since its emergence in the Delmarva Peninsula, DMV1639 has spread to most broiler production areas and has become the most relevant IBV variant in the country due to its negative impact on bird health and performance.

INTRODUCTION

During the winter of 2014, mortality rates as high as 30% due to nephritis were documented in broilers in the Delmarva Peninsula of the United States. From 2015 to present we saw a shifting trend in the prevalence of lesions from severe nephritis to mostly airsacculitis. Economic losses in broilers are now mostly due to airsacculitis condemnations and decreased performance. The virus has also spread to areas with broiler breeders and table egg layers. The emergence of DMV1639 in these long lived birds has been associated with drops in egg production and increased prevalence of false layers.

CLINICAL SIGNS

In the purely nephropathogenic form the main clinical signs are flushing, depression and huddling. Due to wet litter the feathers are markedly soiled. In the respiratory form, there are no overt respiratory signs but the birds develop airsacculitis observed during necropsy or at the processing plant. In laying bids the main clinical sign is decreased egg production. Egg shell abnormalities consistent with IBV are seen when hens are infected while in egg production. The emergence of DVM1639 has been associated with an increased prevalence of false layers. These are ovulating but non-laying hens due to early damage to the oviduct. In some flocks with false layers, egg production has been documented to stall at 68-85%.

GROSS LESIONS

In the acute phase of the purely nephropathogenic form we saw mostly swollen kidneys. In dead birds occasionally we saw pale kidneys with a prominent tubular pattern. The birds were markedly dehydrated. With the current respiratory infections, we see mostly abdominal airsacculitis with suds that progress to fibrinous or caseous exudate. In the case of false layers, the most common lesion is a cystic left oviduct.

DISCUSSION

DMV1639 has demonstrated to be a severe challenge to bird health and performance due to its ability to cause lesions in the urinary, respiratory and reproductive tracts.

A MORPHOMETRICS COMPARISON OF NORMAL POULT INTESTINES WITH OR WITHOUT PROBIOTIC SUPPLEMENTATION

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ABSTRACT

Three hundred twenty one-day old turkey poults were divided into two treatments with five replicates per treatment. The probiotic was administered by spray on day one and in the water day 7 and 14 to Group 2. At days 7, 14 and 21 ten (10) birds were randomly selected and humanely euthanized and 1" section of duodenum and jejunum/ileum were collected and submitted for morphometrics. The duodenum and jejunum/ileum samples were histologically examined and data collected without knowledge of treatment. The results were reported with knowledge of treatment. Minimal differences were detected in the duodenal samples, but in the jejunal samples a difference was identified with regard to surface area.

INFECTIOUS BRONCHITIS VIRUS PREVALENCE, CHARACTERIZATION AND STRAIN IDENTIFICATION IN CALIFORNIA BACKYARD CHICKENS

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SUMMARY

Infectious bronchitis virus (IBV) contributes to respiratory, reproductive and renal disease and in some cases mortality in chickens. California harbors significant numbers of commercial poultry farms as well as numerous small flocks, often closely situated, leading to transmission and exchange of infectious agents via spontaneous and vaccine-associated infections between the two. Here we examined all submissions of backyard chickens to the Davis branch of California Animal Health and Food Safety laboratory system during the first three months of 2019. All cases were analyzed for:

a) Causes of mortality and presence of IBV in the trachea, kidney and cecal tonsils by PCR and immunohistochemistry.

b) Histologic lesions in the trachea and kidney.

c) Sequence data of positive IBV cases.

Of the 120 birds tested, IBV was detected in 18% of backyard chickens tested, where eighteen birds had strong and four had weak positive IBV loads in at least one of the tissues tested. Cecal tonsil tissue was the sample type with the highest percentage of IBV detection. The PCR correlated well with the immunohistochemical results and case data and histopathology revealed that IBV was clinically significant in at least four birds. The IBV strains of eight cases were most similar to CA1737 (91% - 96% identity), two were most similar to Cal99 and ArkDPI with 89% identity, respectively, and five birds had strains that did not have substantial matches to any of the reference strains. Those strains showed the closest relationship to Cal557 (81% - 84% identity).

INTRODUCTION

IBV is a gamma coronavirus that is highly contagious in chickens, primarily affecting the respiratory system. Reproductive tract and/or renal disease can be observed, and death can occur when complicated with secondary infections (1-3). The virus resides in the cecal tonsils post-infection for years (1).

Commercial poultry farms in the US and throughout the world have a high prevalence of IBV, and vaccination is the commonly used management strategy. While raising backyard chickens has become a remarkably common practice, they are typically not vaccinated for IBV and IBV has been the second most commonly detected virus in backyard flocks in the US over the years (4, 5). Information on the prevalence, significance of infection and circulating strains in backyard poultry is not available.

This study plots the prevalence of IBV in backyard chickens submitted to the Davis branch of the California Animal Health and Food Safety Laboratory System, UC Davis, for necropsies and provides information on the pathological and diagnostic features of IBV, the relation of IBV to causes of mortality, and circulating strains in the field.

MATERIALS AND METHODS

Case data. Backyard chickens submitted to the Northern (Davis) and Southern (San Bernardino) branches of the California Animal Health and Food Safety Laboratory System were included in the study. The database (STARLIMS v10) was searched for all backyard chickens submitted to the two laboratories for diagnostic investigations between January 1st and March 31st of 2019. Case reports were analyzed for the locations of the flocks at the county level, and information on age, sex, breed of chicken when recorded, as well as the cause(s) of death and secondary and/or underlying pathological findings. The IBV positive chickens were mapped using a web service mapping tool GeoNames®.

Histology and IBV testing. Two sets of trachea, kidney and cecal tonsil tissues were collected during the time of necropsy; one set fixed in 10% buffered

formalin and cut at 4µm sections for hematoxylin & eosin (HE) staining and IBV immunohistochemistry (IHC), and another set collected in whirl pack bags for freezing at -80°C.

Histopathology was performed on HE sections of trachea and kidneys in every bird with available tissues. The scoring of histological lesions was based on mild (+1), moderate (+2) and severe (+3) lesions based the presence of a) single-cell necrosis in the epithelium, and b) leukocytic infiltrations in the tracheal epithelium and lamina propria in the trachea. The criteria in the kidney were:

a) Tubular necrosis characterized by distention of convoluted tubules, degeneration/coagulative necrosis of the tubules, and sloughed epithelial cells and heterophils in the lumen.

b) Lymphoplasmacytic interstitial infiltrations

c) Gout.

Immunohistochemistry for IBV was performed using monoclonal antibodies described previously (6). Procedures included 3% hydrogen peroxide treatment in water for 10 min after deparaffinization and rehydration of the tissue sections then heating the slides in a decloaker for 10 min, at 121°C for antigen unmasking in Diva Decloaker Solution (Biocare Medical, DV2004G1). For primary antibody, a cocktail of each monoclonal (9.19 and 9.4) was diluted 1:10,000 each in DaVinci Green antibody diluent (Biocare Medical, PD900M), and incubated for 45 min at room temperature (RT). Envision + Anti-Mouse HRP-Polymer (Dako, K4001) was applied to slides as secondary antibody and incubated for 30 min at RT. TBS-Tween was used for rinses between steps. Slides were incubated for 15 min at RT for visualization by AEC chromogen (Dako Ready-to-Use, K3464), counterstained by Mayer's Hematoxylin, blued, and rinsed in deionized water before applying aqueous mounting medium and finally permanent coverslips.

Interpretation and scoring of the immunostaining were conducted on the trachea, kidney, and cecal tonsils. In all tissues, specific intense immunostaining of the cytoplasm of epithelial cells and of mononuclear cells in the lamina propria was considered positive and ranged between focal (+1), multifocal (+2) and extensive (+3) staining for scoring. The scoring was established by the inter-observer agreement of at least three of the authors (AM, OGV, ERB and OEB).

Real-time polymerase chain reaction (PCR) was carried out initially on the pool of fresh frozen trachea, kidney, and cecal tonsil. Total RNA was extracted using a commercial magnetic beads kit (Ambion 1836, Thermo Fisher) following the recommendation of the manufacturer for automatic extraction using a Biosprint 96 well machine (Qiagen). RNA was subjected to standard PCR assay targeting the S1 gene of the viral genome (7). When positive, the tissues were each tested separately by PCR, and the Ct values recorded. A Ct value of 35 was used as the cut-off point, and any value <35 was considered as "positive" (P), and 35-40 as "indeterminate" (I).

Virus isolation was attempted on 25 PCR positive tissues. Tissues were minced with a scalpel, homogenized in viral transport media (VTM) using a Milteny gentleMACS Octo dissociator, diluted in VTM to a concentration of 1:10 weight/volume, and syringe filtered through a 0.2-micron filter. Two hundred µL of filtered homogenate from kidney and tracheal swabs were inoculated into Specific Pathogen Free chicken eggs (Charles River) and incubated for up to 5 days.

Standard PCR and phylogenetic analysis kidney and/or cecal tonsil tissues with Real-time PCR Ct values of <32 were subject to standard PCR targeting the S1 gene (8). For PCR amplification, the QIAGEN One-Step RT PCR kit was used following the manufacturer's instructions. The resulting amplicons were run on a 4% agarose gel, cleaned up using Amicon Ultra (Millipore Sigma) 0.5 mL centrifugal DNA filters, and sequenced by the Sanger method. Forward and reverse sequences for each sample were aligned using the program Geneious Prime (version 2020.0.5, Biomatters Inc). Multiple sequence alignment of the IBV S1 gene fragment was produced using the MUSCLE alignment algorithm and a phylogenetic analysis using a neighbor-joining method performed.

RESULTS

Case data. A total of 136 case submissions to Davis with 144 backyard chickens and 57 submissions to the San Bernardino branch with 60 chickens were found in the database. From the Davis cases, fresh and formalin-fixed tissues were not collected for testing in 24 birds and were discarded from further analysis.

From the 120 chickens submitted to Davis, the majority were females (n=89), with 19 males and 12 chickens with unidentified sex. A wide range of ages and breeds were observed when stated in the reports. Breeds of those positive for IBV by PCR were available in eight reports and consisted of Silkie chickens (n=2) and one Cornish hen, Buff Orpington, Marans, Brahma, Araucana, and Jersey Giant each.

The cases were submitted from Northern California districts Modesto and Redding except for two cases submitted from southern California, one chicken from Los Angeles County and one case with two chickens from Ventura County. The location distribution of the birds positive for IBV by PCR is shown in Figure 1.

PCR, IHC and histopathology. Eighteen of the 120 chickens (15%) had substantial (Ct <35) viral

loads in the pooled trachea, kidney, and cecal tonsil while four birds had very low/indeterminate ("I"; Ct >35) values. The IBV PCR and IHC on each tissue (trachea, kidney or cecal tonsil) as well as HE lesion scores in trachea and kidney on those birds with positive and indeterminate Ct values are given in Table 1. The initial pooled sample was positive in bird no. 1 however PCR was negative on individual tissues, likely due to the dilution effect and low viral loads. Trachea was the only tissue available for PCR in bird no. 18 and was positive with a high viral load (Ct=23) and concurrent infectious laryngotracheitis virus infection, the kidney and cecal tonsil were not available for testing in this bird.

Of the sixteen birds in which individual PCR analysis was available, trachea was positive in four, kidney in twelve and cecal tonsil was positive in every bird (Table 1). Of the four birds with indeterminate values in the pooled samples, all were negative in the trachea while three were positive (nos. 19, 20, 21) and one (no. 22) had very low very high Ct in the cecal tonsil. When available, IHC results correlated with the positive specimens, and the cecal tonsil was not available for IHC in the one "I" case.

Virus isolation and sequences. None of the IBV positive cases had a growth in SPF chicken eggs. Sequences from fifteen cases with kidney and/or cecal tonsil tissues with Ct values of <32 were obtained and analyzed phylogenetically (Figure 2). The largest cluster of eight cases were most similar to CA1737 (91% - 96% identity), D1901639 was most closely related to Cal99 (89% identity), D1903620 was most closely related to ArkDPI (89% identity), while five cases (bird nos. 3, 4, 8, 11, 13) did not cluster closely with any of the available reference or vaccine strains. The closest match to this cluster was Cal557 (81% - 84% identity). A BLAST search of the GenBank database showed a similar lack of similarity to any other published IBV sequences. Cal557 (FJ904715) and a strain from Georgia (KM660630) were the most similar matches with an identity of 84% and 86%, respectively.

CONCLUSION

Among the analysis of tissue distribution, cecal tonsil was the sample type with the highest percentage of IBV detection. Genetic analysis of the detected IBV cases clustered to more than one variant, primarily CA1737 (eight cases), which has been previously detected in broiler chickens in California.

ACKNOWLEDGMENTS

We would like to thank Karen Sverlow and her team at the CAHFS histology laboratory for their expertise and extensive work.

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Figure1.

Figure 2. The sequence identities of IBV detected in seventeen tissues of fifteen backyard chickens from California.

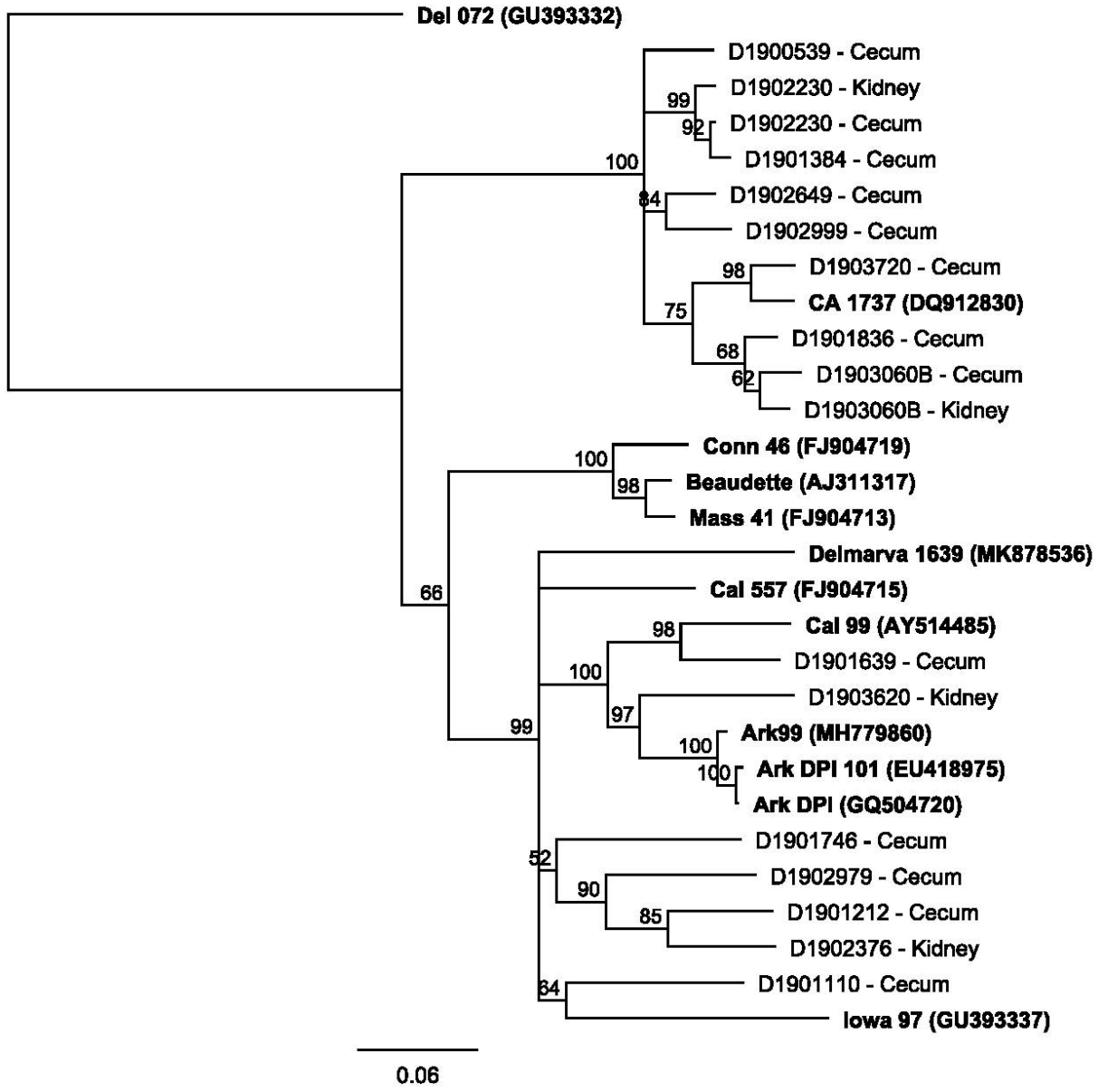

Table 1. Infectious bronchitis virus PCR positive backyard chickens in trachea, kidney and/or cecal tonsils, with IBV IHC and histology scores.

					PCR			IHC			HE – T		HE – K			
Chicken	Sex	Age	COD	Primary Dis	T	K	C	T	K	C	Infl	Nec	Infl	Nec	Dil	Gout
1	F	5y	EU	Tracheitis, SPS	-	-	-	-	+2	-	+1	-	-	-	-	-
2	F	3y	SD	Carcinomatosis	-	I	+	-	-	-	+1	-	-	-	-	-
3	F	9m	SD	LPD, histological, Coelomitis	-	+	+	-	+2	+1	-	-	-	-	-	-
4	F	1y	U	LPD, histological, Visceral gout	-	+	+	-	+1	+1	+1	-	+2	+3	-	+3
5	M	7m	SD	Renal disease, Visceral gout	+	+	+	-	+1	+1	-	-	+1	+2	+1	+2
6	F	U	U	Respiratory disease	+	I	+	-	-	S	+3	-	-	-	-	-
7	F	8.5m	U	LPD, visceral	I	+	+	-	+1	+2	+2	-	-	+1	-	-
8	M	Ad	EU	LPD, histological, Respiratory disease (MS)	-	+	+	-	+1	S	+1	-	+2	+1	-	-
9	F	15m	EU	LPD, histological	-	+	+	-	-	+3	+1	-	-	-	-	-
10	F	Ad	U	Pulmonary granulomas	-	+	+	-	+2	-	+2	-	-	-	-	-
11	F	10m	SD	Renal disease, Visceral gout	+	+	+	-	+3	+1	-	-	+1	+2	-	+2
12	F	3m	SD	Respiratory disease	I	+	+	-	+1	+1	-	-	-	-	-	-
13	F	5m	EU	LPD, visceral	I	-	+	+1	-	+1	+3	-	+1	-	-	-
14	F	U	SD	Renal disease, LPD, histological	I	+	+	+1	+3	NA	-	-	+2	+2	+1	+2
15	F	3y	EU	LPD, histological, Respiratory disease	+	+	+	+1	-	+2	+3	-	+1	+1	-	-
16	U	U	U	LPD, histological, Systemic bacterial infection	-	+	+	-	+2	S	+1	-	-	-	-	-
17	F	Ad	EU	LPD, histological	I	I	+	-	-	+2	+1	-	+2	+1	-	-
18	F	2y	SD	Respiratory disease (ILT)	NA	NA	NA	-	-	-	+3	+3	-	-	-	-
19	M	6m	U	Respiratory disease	-	-	+	+1	-	+2	+2	+1	-	-	-	-
20	F	8y	EU	Neoplasia, sarcoma	-	I	+	-	+2	+2	+1	-	-	-	-	-
21	F	4.5m	SD	Anemia, Ectoparasitism	-	-	+	-	-	-	-	-	-	-	-	-
22	M	10m	EU	Respiratory disease (*O. rhinotracheale*)	-	-	I	-	-	NA	+1	-	-	+	-	-

Abbreviations: F: female, M: male; y: year, m: month, COD: cause of death; U: unknown, EU: euthanized, SD: sudden death; LPD: lymphoproliferative disease; MS: *Mycoplasma synoviae*; ILT: infectious laryngotracheitis; T: trachea, K: kidney, C: cecal tonsil, NA: not available; I: indeterminate; Infl: inflammation, Nec: necrosis; Dil: dilation.

SPOTTY LIVER DISEASE IN U.S. LAYERS AND GENETIC ANALYSIS OF *CAMPYLOBACTER HEPATICUS*

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SUMMARY

Campylobacter hepaticus is a causative agent of spotty liver disease (SLD) resulting in significant health issues and productivity losses in layers but often overlooked. From 2018-2019, we have received clinical samples from suspected cases of SLD. Its characteristic lesions, whitish-grey spots, on the surface of livers were identified during necropsy and liver and bile were collected aseptically. *Campylobacter hepaticus* was successfully isolated and identified from the samples. After confirmatory bacteriological tests, whole genome sequencing (WGS) analysis was performed to identify multiple putative virulence factors and to characterize the isolates. Sequence analysis showed six clusters of the isolates separated by geographical regions.

INTRODUCTION

Spotty liver disease in the U.S. has been reported since the 1950s and was then known as avian vibrionic hepatitis cause by a vibrio-like organism. In 2015, Crawshaw, T. R. *et al*. isolated a novel *Campylobacter* sp., characterized the bacteria, and reproduced SLD in experimentally infected chickens (1). *Campylobacter hepaticus* was officially described 2016 (2). The disease is characterized by multifocal liver spots white to grey 1-2 mm in size (1). It contributes to increased mortality and significant decline of egg production beginning at peak egg lay (3). The organism is fastidious and can be difficult to isolate due to its extended incubation requirements making it easily overgrown. Since December of 2018, the Scientific Support and Investigation Unit (SSIU) at Ceva Animal Health, Lenexa, KS has received eight diagnostic cases in attempt to isolate and characterize *Campylobacter hepaticus*. These cases presented with sudden onset at 23-28 weeks of age, mortality increases of up to 10% for several weeks, associated fever with enteritis/diarrhea and up to 10% drop in egg production.

MATERIALS AND METHODS

Bacterial culture. About 1 mL of bile fluid or 1 g of aseptically macerated liver was added to 9 mL of modified Preston Broth. Cultures were incubated at 39-41°C for up to one week in 8-10% CO2 incubator. At three and five days post inoculation, bacteria were isolated on Brucella Agar plates containing 5% horse blood (Thermofisher, Waltham, MA). Agar plates were incubated similarly for up to 14 days. Suspect *Campylobacter hepaticus* colonies are pinpoint to small, grey, circular, and have a convex profile with entire margins. They may have the typical *Campylobacter* dewdrop appearance. Bacterial cultures were tested using conventional bacteriological methods and the API® Campy identification system (bioMerieux, France). Suspect *Campylobacter hepaticus* isolates were frozen for storage in tryptic soy broth (Sigma-Aldrich, St. Louis, MO) with 25% glycerol.

Whole genome sequencing. DNA was extracted from isolates or directly from tissue samples (or bile/liver) and sequenced on an Ion Torrent S5 sequencer. Sample raw reads were trimmed and curated with FASTP (4). Trimmed data were assembled *de novo* with SPADES (5). Aligned genomes were verified to be *Campylobacter hepaticus* with KRAKEN2 (6). Resistance and virulence genes were identified with virulence factor database (VFDB) database (7) using ABRICATE (8). A Virulence gene clustering dendrogram was built in Bionumerics (9) analyzing the virulence gene similarity to the reference strain *Campylobacter jejuni* ATCC 700819.

Invasion assay. The cellular invasion of *Campylobacter hepaticus* isolates was compared to *Campylobacter jejuni* ATCC 700819 and *Camplylobacter hepaticus* HV10 using chicken hepatocellular carcinoma cells (LMH). Van, T. T. H., *et al*. showed differences in LMH invasion by *Campylobacter* species10. It was the goal to repeat this data and determine if there was a difference in invasion of *Camplylobacter hepaticus* isolates by geographical region. Bacterial culture was grown on Brucella Agar plates containing 5% horse blood for 48 hours in the atmospheric conditions described in a previous study (9). Bacterial culture suspensions were

prepared in Brucella Broth to an OD_{600nm} of 0.1 roughly 108 cfu/mL. The LMH cells were planted in 24-well tissue culture plates to form monolayers and when they reached 90% confluency, it was inoculated with 1 mL of each isolate in triplicate. After inoculation, the cells were incubated at 37ºC in 5% CO2 for five hours and washed twice with PBS and treated with 1 mL of DMEM with 400µg/mL gentamycin. Following addition of gentamycin, the cells were incubated at 37°C in 5% CO2 for 90 minutes. The cells were washed three times with PBS and lysed with 200µL of 0.3% triton-X for 10 minutes at 37°C. The cell lysate was further diluted with an additional 800µL of Brucella Broth and incubated at 37°C in 5% CO2 for 15 minutes. Plate counts were conducted on the initial inoculum and recovery following cell lysis and the percent invasion calculated.

RESULTS AND DISCUSSION

Bacterial culture. A total of eight cases were received consisting of 76 bile samples and 31 liver samples. Twelve *Campylobacter* hepaticus isolates were successfully cultured and identified from four cases. The phenotype characteristics shared by these isolates were as follows; catalase positive, oxidase positive, gram negative wavy rods. All isolates were urease negative, reduced nitrate, and possessed the enzymes esterase and gamma glutamyl transferase. Isolates from the Midwest were hippurate positive and alkaline phosphatase negative while the opposite was true for isolates from the South. The API Campy identification system doesn't specifically identify *Campylobacter hepaticus*; these isolates were distinguished from *Campylobacter jejuni* by succinate and malonate assimilation. These reactions were mostly positive for *Campylobacter jejuni* and negative for *Campylobacter hepaticus*.

Whole genome sequencing. From the submitted cases, a total of 27 genome sequences were obtained including 12 from *Campylobacter hepaticus* isolates and 15 sequences obtained from metagenomic analysis of DNA directly obtained from the bile and liver samples submitted. *In-silico* analysis of the B subunit of the *Campylobacter* invasion antigen (ciaB) gene was performed. A secretory protein encoded by the ciaB gene plays a critical role in the invasion of chicken epithelial cells and deletion of ciaB gene resulted virulence reduction in the case of *Campylobacter jejuni* infection (11). Figure 1 shows analysis of the ciaB gene present in 25 of the *Campylobacter hepaticus* sequences in comparison to the references for *Campylobacter jejuni* NC002163.1, *Campylobacter coli* CP019977.1, and *Campylobacter hepaticus* CP031611.1. This maximum parsimony dendogram shows two distinct geographic clusters of isolates based on ciaB gene sequence similarity. There are 517 and 413 single nucleotide polymorphism (SNP) differences between *Campylobacter jejuni* reference and the Midwest cluster and the Southern cluster of *Campylobacter hepaticus* respectively. Between the two *Campylobacter hepaticus* clusters there are 366 SNP differences.

A number of putative virulence genes were assessed and compared to the 3225 virulence genes in the VFDB database (7). This database contains known putative virulence factors across bacterial species. In this analysis, our focus was to utilize diversity of shared putative virulence genes by comparison. Of 3225 virulence genes, 31 genes were present in all sequences and are shown in Figure 2. This cluster analysis continued to show geographical distinction between Midwest and Southern isolates but also further separation in the Southern Cluster generally by state.

Invasion assay. Five isolates were assessed for LMH invasion. Three were from diagnostic cases, chosen by differing geographic location, and two reference strains *Campylobacter jejuni* ATCC 700819 and *Campylobacter hepaticus* HV10. The invasion results were lower than expected (data not shown) compared to Van, T. T. H., *et al*. (10) who reported higher invasion percentages with *Campylobacter hepaticus* compared to *Campylobacter* j*ejuni* and an overall higher invasion percentage for all isolates. The differences we observed may be due to the higher inoculum volume used for invasion, 1.0 mL compared to 0.1 mL. The lower cellular surface area to inoculum volume may have affected the invasion assay results. The testing will be repeated to verify our findings.

CONCLUSION

Campylobacter hepaticus and SLD are showing a re-emergence in the United States. Laboratory analysis requires specific methods for culture and isolation. No commercial vaccines are available to combat SLD but autogenous vaccination may be an option. The case histories described here indicate that diagnosis by traditional methods can distinguish *Campylobacter hepaticus* from other *Campylobacter* species. Molecular methods continue to offer insight into SLD and provide characterization of *Campylobacter hepaticus* isolates.

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Figure 1. Maximum parsimony tree of the 1836 bp ciaB gene.

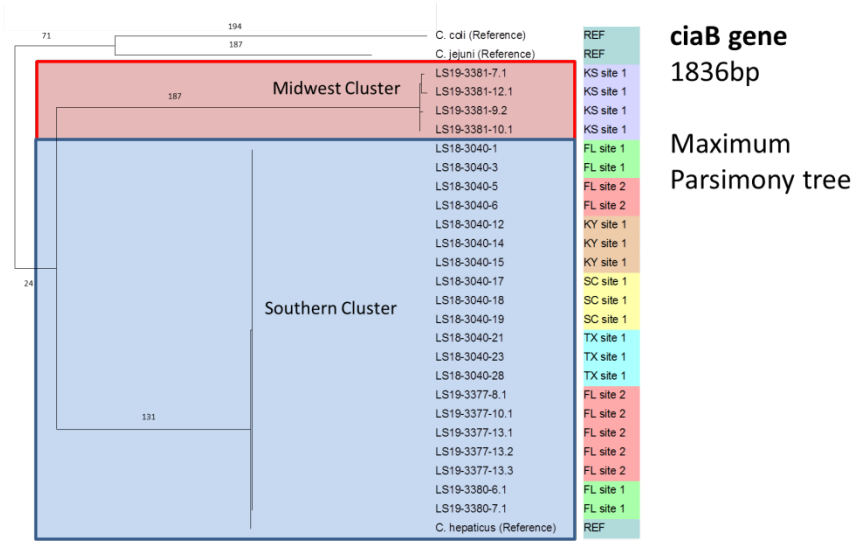
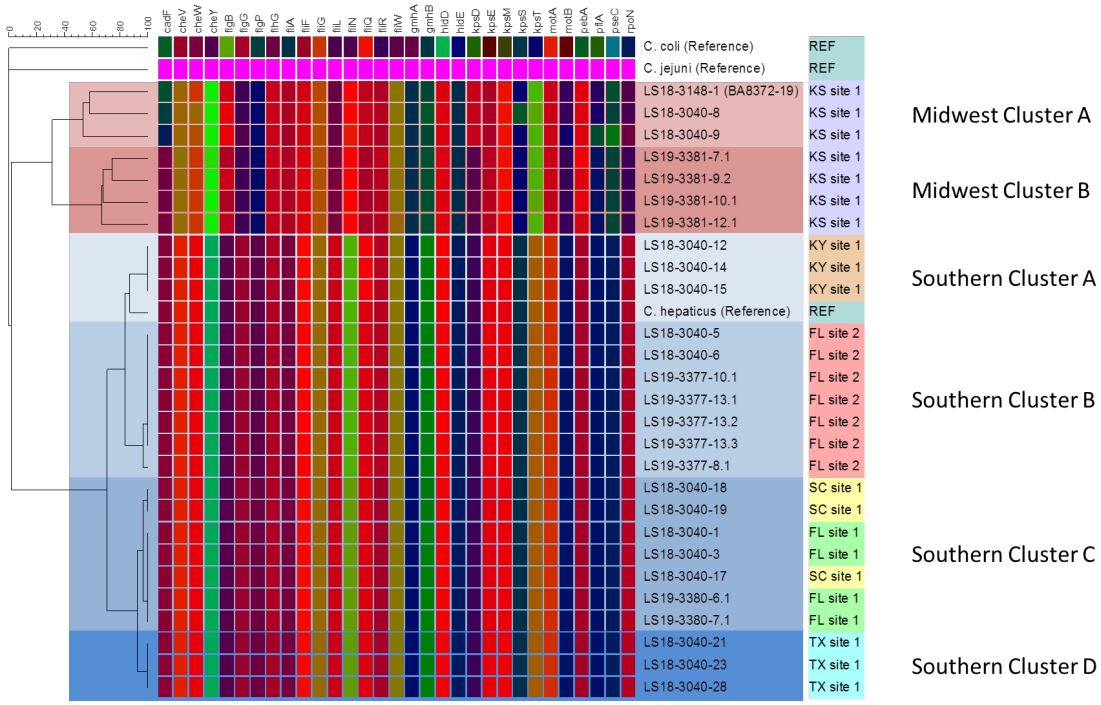


Figure 2. Cluster analysis of 31 putative virulence factors present in all sequences.



FIELD STRAINS OF *MYCOPLASMA GALLISEPTICUM* IN SPAIN WITH TS-11 *MGC*2 SEQUENCE

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SUMMARY

Laboratory tests using *mgc*2 identified *Mycoplasma gallisepticum* strains in Spain circulating with 100% sequence similarity (5) with the MG vaccine ts-11. There are other MG field strains in Genbank with this sequence but the parent flocks of clinically affected broilers were assumed to have ts-11-like infections (4). Further characterization with melt-MAMA (7) and MLST (3) showed that the isolates were not derived from ts-11. Other isolates in non-vaccinated flocks had similar *mgc*2 results. Single gene analyses are not suitable for DIVA tests.

INTRODUCTION

Around the world many laboratories are offering strain identification of *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS). There are a lot of GTS (gene targeted sequencing) PCR based tests (and some other variants including HRM) described and laboratories without mycoplasma experience or back up often select a target on little local knowledge. Partial *mgc*2 sequencing has been popular in many parts of the world and misinterpretation of the results has sometimes tainted the reputation of ts-11.

MATERIALS AND METHODS

Breeder farms in Portugal used ts-11 in their vaccination program at 18 weeks of age (before transfer). No antibiotics were routinely used after vaccination. Eggs were shipped to Spain for hatching. Breeder and broiler flocks were examined. Some broiler flocks had clinical respiratory disease.

Initial testing of a progeny broiler flock with clinical respiratory disease by PCR by a Spanish laboratory demonstrated MG and *mgc*2 sequencing (5) on tracheal samples suggested that the MG could be related to ts-11 (100% similarity). A survey was then carried out where choanal cleft swabs from birds (breeders and broilers) were transferred to FTA cards.

RESULTS

In August 2019: Comparison of the sequences showed that *mgc*2 sequences obtained from the three tested Spanish MG strains and the ts-11 vaccine strain showed identical nucleotide sequences and were different from the strains ATCC 19610, F, R(high), R(low) and S6. However, when the obtained 274 bp long fragment of the *mgc*2 gene was submitted for NCBI BLAST search, they showed 100% identity with several wild-type MG *mgc*2 gene sequences available at Genbank.

Melt-curve analysis of mismatch amplification mutation assays (melt-MAMAs) targeting the point mutations of the *plp*A, *glp*K, and *pot*C genes of MG were used in order to detect and differentiate ts-11 vaccine strain and wild-type MG isolates (7). The samples proved to be wild-type MG based on the melt-MAMAs.

Multi-locus sequence typing (MLST) was performed based on six housekeeping genes (*atp*G, *dna*A, *fus*A, *rpo*B, *ruv*B and *uvr*A) (3) in order to genetically characterize the Spanish MG strain. The strain showed unique sequence type (ST), and was closely related to ST24 and ST8 (belonging to strains from Spain, Ukraine and Italy). Thus the Spanish MG strain was identified as a wild-type strain which is very distantly related to strain ts11.

DISCUSSION

Clinically diseased broilers from a ts11 vaccinated parent flock were shown to be infected with MG strains with *mgc*2 sequences 100% similar to ts-11 raised the question of vertical transmission of ts-11 to progeny similar to the case reported in Georgia (4). Further testing using melt-MAMA DIVA (7) testing and confirmed by MLST (3) showed the MG strains infecting the broilers were not related to ts-11.

Kleven (6) warned against the use of mcg2 sequences for definitive strain identification of MG strains and suggests that the technique could be used as a preliminary identification, but diagnostic labs need to put out results that are useful. This is particularly problematic where the laboratory does not

have access to mycoplasma expertise and in fact in this situation where the result could lead to field strains being identified as progeny of ts-11 vaccination. Preliminary identification implies that other tests are necessary. The only test result that would have been definitive in the broilers with *mgc*2 sequencing was that the MG strain was different from ts-11. If it is not different then alternative testing is needed to show that the strains are closely related.

In the UK (1) and Spain there have been serious misinterpretation of laboratory results using just *mcg*2 sequences. The vaccine ts-11 has been suspected of vertical transmission like described in El Gazzer and others (4) and indeed the term ts-11-like had been used throughout Spain with the results of the *mgc*2 sequencing with suspicions of contamination of unvaccinated layer flocks. With the reported information presented here there is no evidence of ts-11 like strains in Spain and the main conclusion is that there are field strains in Spain MG field strains with *mgc*2 sequences identical to ts-11.

The usual performance of ts-11 in breeder flocks for the life of the flock is as described in Barbour and others (2). The protection of the breeder flock from MG field strain infection and the prevention of vertical transmission of MG. There may be maternal antibody and this is proportional to the antibody in the breeders.

The role of MS in the respiratory disease seen here also needs to be considered. MS was demonstrated in the parent flock and the broilers.

CONCLUSION

The use of *mgc*2 testing can only be justified to show two MG strains are different. Melt-MAMA (cheap and fast) and MLST allow correct identification that the field strain was not related to ts-11.

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DIVA TESTING OF FLOCKS VACCINATED WITH LIVE *MYCOPLASMA* VACCINES

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SUMMARY

Making valid conclusions from molecular tests on URT swabs as to the effectiveness of live mycoplasma vaccination and predicting whether flocks are protected are current problems for clinicians. Problems range from *Mycoplasma gallisepticum* infection kinetics with vaccine and field strains populations dropping to around the level of detection after 10 weeks, (further complicated as many DIVA PCRs are less sensitive than species qPCRs) to problems with interpreting whether mixed infections are real or laboratory artefact when a single SNP is the basis of the test. Eventually we will move to WGS but this too may have problems especially where the sample is not a pure culture. In the meantime we need a new generation of DIVA tests that test more than just one SNP.

INTRODUCTION

Vaccination with live mycoplasma vaccines complicates monitoring for field strain infections. There are also problems with how to confirm vaccination. Serological monitoring becomes largely useless after vaccination and arguably a waste of money (8). Although we do not have laboratory tests for all poultry vaccines to monitor effective vaccination (for example coccidiosis vaccination and Marek's disease vaccination) it would be desirable to develop some routine form of monitoring to monitor mycoplasma vaccinated flocks.

Timing of sampling after vaccination is important. The kinetics of mycoplasma vaccine populations are poorly defined especially if we consider the availability of qPCR (why has no-one done some systematic longitudinal studies to date?). From pathological states MG and MS can be easily demonstrated by PCR and cultured early on (especially if sampled before effective antibiosis) but in the chronic infection MG especially can be particularly difficult to demonstrate and even harder to culture. MS populations in the URT usually are easy to demonstrate but isolation may be complicated by commensal mycoplasmas and acholeplasma infections or suboptimal media. Seemingly MG infections may become easier to demonstrate after 40 weeks of age in healthy birds. MG live vaccine populations seem to have similar kinetics (Noormohammadi and Whithear 2019).

Many DIVA PCRs and GTS based tests have been developed. Initially the targets were sequences that were available and showed variation between strains. Unfortunately, these sequences were often surface proteins (for example *vlh*A) which interacted with the hosts immune system or sometimes SNPs that had be characterized as potential attenuating mutations by comparison of the live mycoplasma vaccine and the parent strain (for example the obg gene in MS vaccine MSH). Both these tests are potentially unstable due to fitness pressure *in vivo*.

This paper surveys the authors experience with current DIVA testing

MATERIALS AND METHODS

Pooling of birds (two or more birds per swab) and swabs (up to 5 per PCR pool) was commonly used. Most assessments had look at between 2 and 10 PCRs per flock (30 birds maximum).

M. synoviae. Usually a pan MS PCR was used first (10) before moving onto DIVA PCR. Only positive samples were subjected to DIVA PCR in this situation. GTS of *vlh*A genes for strain ID was been done according to the methods of Hammond and others (3) and analyzed by sequence similarity directly from URT swabs (usually choanal cleft). Sequences were compared to reference sequences of MSH. Other systems tested included obg gene based SNP including melt-MAMA. Anicon MS DIVA PCR results were also reviewed on a small number of samples.

The 56 bp duplication in MS1 (MSD) was used in Thailand to differentiate vaccine strains from field strains in vaccinated breeders (Dijkman and others 2014). PCR product size was visualized on an agarose gel.

***M. gallisepticum*.** PCR tests The PCR tests described by Sprygin and others (10) was used. See paper on *mgc*2 GTS analysis for identification of ts-11 (5)

RESULTS

M. synoviae. The *vlh*A sequences were usually easy to interpret. Over 500 samples were analysed with reads in both directions being synthetized into a consensus sequence before alignment. All insertions or deletions were a multiple of three codons and considered to be one genetic change. This was probably so that the downstream functional parts of the expressed proteins remained in frame.

M. gallisepticum. The biggest problem with MG testing was that the mucosal populations seemed to decrease after 8 weeks till after the peak of lay. This made detection in this period difficult and made valid DIVA tests hard to perform on tracheal swabs (Table 1).

DISCUSSION

Using FTA papers was preferred but the impression was that we were probably losing some sensitivity over swabs. The advantages because of ease of transport, inactivation of infectious agents and stability were considered to outweigh the downside of some loss of sensitivity and this could be compensated by taking more samples. The effect of pooling samples was considered to be dilution of positive material in other material gathered,

M. synoviae. We did not have the problem of field strains not amplifying with Hammond primers. (samples were mainly from western Europe). Confirmation of colonization after vaccination was usually with samples at 6 weeks after vaccination with MSH and 8 weeks with MS1. MSH and MS1 MAMAs have also been described (4)

Failure to amplify was usually associated with reduced sensitivity of DIVA PCRs on the same DNA prep from an URT swab were approximately 102-3 times more copies were needed for a DIVA PCR test to work compared to a pan MG/MS PCR test.

Despite all this there are still problems with using *vlh*A sequence typing or obg allele characterization for the identification of MSH progeny in (vaccinated) birds. This has been highlighted by a study (6) on MSH vaccinated flocks placed on a remote site identified some MSH re-isolates (confirmed by MLST) as having new *vlh*A and obg alleles that would not allow correct categorization of them by these later tests. It may also be the origin of some test results claiming that field strains are also in samples that are predominantly vaccine.

M. gallisepticum. There seems to be a definite need for tests that will confirm vaccination with ts-11 (and MSH) and protection (including exclusion of field strains). At the moment the only way that protection can be effectively assessed is by transporting some birds to the laboratory and challenging the birds with MG in the lab. This has been done in the past for assessment of immunity in birds from flocks that have had no or minimal serological response to vaccination (8) but is not practical in the field.

In the absence of any other information we believe that if the vaccine can be demonstrated as colonizing the bird then it is reasonable to assume that the bird is protected (from disease or infection). Studies on flocks at the time of moulting showed that 93% of MSH vaccinated breeder flocks (n>600) were colonized. This is without troubleshooting (poor vaccination or antibiotic treatment for MG infection).

The duration of immunity from ts-11 vaccination has been questioned (for example Hyline manual) as 40 weeks post vaccination. This is despite previous publications showing protection without an endpoint at 57 weeks. Increases in serological responses in ts-11 vaccinated flocks after peak of lay without demonstration of field strains (12) or clinical problems suggests that this may be a normal occurrence and it coincides with increased ease of demonstration of ts-11 in the URT of these vaccinated hens after long periods where neither vaccine or MG field strains could be detected. This myth that ts-11 immunity wans around 40 weeks has spawned inconclusive investigations like Vance and others (11).

Some new MG DIVAs have recently been described for all the available MG vaccines (9) and Bekő and others (1). These have the advantage of targeting SNPs that are not under fitness or immune pressure.

CONCLUSION

MAMA PCRs are an attempt to make DIVA testing possible in a simple PCR format which may be more accessible in company laboratories. There are now MAMAs for all MG and MS live vaccines but they have the problem of all DIVA PCRs of a drop in sensitivity compared to pan species PCR.

The concept of preliminary DIVA test when it comes to identification of mycoplasma vaccine strains in vaccinated flocks is particularly problematic. Local MG field strains can have the same *mgc*2 sequence. Vaccine strains may no longer have vaccine alleles (MS *vlh*A) or temperature sensitive phenotype or genotype.

There is a need for new tests that are not targeting sequences whose products are under immune or fitness pressure. Perhaps modification of MLST tests that are only asking is it vaccine or field strain. Or perhaps WGS. But more than this there is a need for any new test to be tested on field samples (from sites like the

one used by Moronato) before it is released into the field.

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Table 1. Farm survey of flocks vaccinated with ts-11 and MSH tested by PCR for the presence of the vaccine.

Flock	Age (W)	Vaccination age	Pan MG	MG Diva (Vac/tested)	Pan MS	MS Diva (Vac/tested)
Shed 63	3	Not vacc.	0 of 5	None to test	0 of 5	None to test
Shed 62	14	8 w	2 of 5	0 of 2	5 of 5	5 of 5
Shed 64	15	8 w	3 of 5	2 of 3	5 of 5	5 of 5
Shed G1	15	6 w	3 of 5	3 of 3	5 of 5	5 of 5
Shed 58	22	8 w	1 of 5	0 of 1	5 of 5	5 of 5
Shed 54	34	8 w	2 of 5	0 of 2	5 of 5	5 of 5
Shed G5	34	6 w	0 of 5	None to test	5 of 5	5 of 5
Shed 61	53	8 w	5 of 5	2 of 5	5 of 5	5 of 5
Shed 56	64	8 w	4 of 5	3 of 4	5 of 5	5 of 5
Shed 57	64	8 w	5 of 5	5 of 4	5 of 5	5 of 5
Shed 55	71	8 w	4 of 5	3 of 4	5 of 5	5 of 5
Shed 59	73	8 w	4 of 5	2 of 4	5 of 5	5 of 5
Shed 60	73	8 w	5 of 5	2 of 5	5 of 5	5 of 5
Shed G4	74	6 w	5 of 5	3 of 5	5 of 5	5 of 5
Shed G2	77	6 w	5 of 5	4 of 5	5 of 5	5 of 5

HIGH DIETARY ZINC GLYCINATE CONCENTRATION ON HOST IMMUNE RESPONSE ON NECROTIC ENTERITIS IN BROILER CHICKENS

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SUMMARY

A study was conducted to determine the effects of zinc glycinate at higher concentrations beyond the current recommendation at 100 mg/kg in chicken diets on necrotic enteritis infection. Zinc glycinate at concentration at or above 120 mg/kg decreased mortality and gross lesions of the birds. We did not observe major differences in cytokine responses in the cecal tonsils and spleen post-infection. Cecal tonsils Zn/Cu-superoxide dismutase was reduced by zinc glycinate at 140 mg/kg. This validates the potential of zinc glycinate to alleviate the infection.

INTRODUCTION

Zinc is involved in immunity as co-factors of Zn/Cu-superoxide dismutase and thymulin, and as the structural role in the zinc-finger motif of transcription factors (3). In chicken diets, zinc is currently fed at 100 mg/kg (1). Zinc glycinate is an organic zinc form that improves retention in tissues compared to the inorganic forms (6). We previously demonstrated that zinc glycinate at this concentration alleviated necrotic enteritis infection in chickens (manuscript prepared). However, the biological role on immunity was unclear. Therefore, we conducted this study to determine the benefits of increasing zinc beyond the current recommendation and elucidate the effects on immunity. We hypothesized that zinc glycinate at high concentration will alleviate necrotic enteritis infection in broiler chickens by upregulation of cellular immunity.

MATERIALS AND METHODS

Animals. In experiment 1, day-old Cobb 500 off-sex male by-product broiler chicks were randomly assigned to zinc glycinate treatments at 40, 80, and 120 mg/kg. In experiment 2, the chicks were randomly assigned to zinc glycinate treatments at 100, 120, and 140, and 160 mg/kg. Each treatment was replicated in six pens with eight birds per pen (n = 6). A priori power analyses were carried out utilizing effects size from a similar study of organic minerals on immune status in chickens (4) to determine the sample size required to achieve 97% power (G*Power ver. 3.0.10) (2). The birds were housed in battery cages under standard animal husbandry practices. Necrotic enteritis was induced by oral gavage of 5,000 oocysts of *Eimeria maxima* on day 14, and 108 CFU/mL of *Clostridium perfringens* daily on days 19, 20, and 21. On day 21, birds were euthanized for analyses.

RNA isolation and gene expressions. Total RNA from cecal tonsil, spleen, and jejunum tissues were isolated using the TRIzol/chloroform method. Briefly, tissues were homogenized using the TissueLyser LT (Qiagen, MD, USA) and 5 mm stainless steel beads in TRIzol for six minutes. Chloroform was added to the homogenate and centrifuged to fractionate the total RNA in the aqueous phase. The total RNA was precipitated in isopropanol, washed in 70% ethanol, and then resuspended in molecular-grade water. Optical density at 260 nm was used to determine RNA concentrations using the Epoch Microplate Spectrophotometer (BioTek, VT, USA). The RNA quality and purity with 260/280 and 260/230 above 2.0 and 1.8, respectively, were used. Reverse transcription (RT) of mRNA from total RNA (2 μg) to cDNA was performed in a 20 μL reaction volume containing buffer (50 mM Tris-HCl at pH 8.3, 75 mM KCl, 3 mM MgCl2, and 10 mM DTT), 10 mM DTT, 0.5 mM dNTP, oligo(dT)15 primer, 8 units RNAsin, and 100 units M-MLV reverse transcriptase (Promega, WI, USA) according to manufacturer's guidelines. RNA was first heated at 70°C for 12 minutes and then maintained at 10°C before reverse transcription. The RT cycles were performed in the conditions as followed: 40°C for 1 hour and then 95°C for 10 minutes. Relative gene expressions of pro-inflammatory cytokines (IL-1β, IFN-γ, and TNF-α), anti-inflammatory cytokines (IL-10 and TGF-β) of mRNA from cecal tonsil and spleen tissues were analyzed using the CFX-96 and CFX Maestro (Bio-Rad, CA, USA). Relative gene expressions of jejunum tight junction proteins (Claudin-2 and zona occludin)

and zinc importer (ZIP-9) were analyzed. Expressions were normalized to β-actin as the housekeeping gene, calibrated to expressions of zinc sulfate 40 mg/kg treatment, and reported as fold-change ± SEM. Primer sequences and references are listed in Table 3. PCR reagents and reaction conditions were optimized for each gene. Fold change from the reference was quantified as 2^(-ΔΔCq), where E is the efficiency and Cq is the threshold cycle defined as the cycle number when the qRT-PCR product fluorescence reaches exponentially above the background (5). Expressions were corrected for primer efficiencies as Cq × [log(1+E)].

Flow cytometry. Spleen tissues were homogenized into single-cell suspensions in RPMI using 70 µm cell strainers. Lymphocytes were separated using Histopaque-1077. Cells were plated at a density of 106 cells/mL and then stained for T helper cells using CD4 FITC-conjugated anti-CD4 (1:300 v/v in buffer) (Oxford and Selvaraj, 2019; Shanmugasundaram and Selvaraj, 2011). After staining, the cells were washed and fixed using 0.5% paraformaldehyde in PBS, and then washed before analysis. Flow cytometry data were acquired by CytoFLEX (Beckman Coulter, Inc., Brea, CA) and data were analyzed with FlowJo v9 (Treestar, Inc., San Carlos, CA).

Statistical analysis. Intestinal lesion scores were analyzed by Kruskal-Wallace non-parametric test with posthoc Tukey's HSD. Mortality was analyzed by Chi-square test with posthoc Mann Whitney U test. Gene expression and flow cytometry data were analyzed using one-way ANOVA using SAS v.9.0 (SAS Institute, Inc., NC, USA) to determine the effects of zinc concentrations. When the main effect was significant ($P < 0.05$), the differences between means were analyzed using Tukey's HSD posthoc analysis.

RESULTS

In this study, mortality from necrotic enteritis (**$P < 0.01$) and intestinal gross lesions (**$P < 0.01$) of the chickens on day 21 was decreased by the inclusion of zinc above 120 mg/kg (Figure 1). We analyzed cytokine responses in the spleen as an indication of systemic infection and in the cecal tonsil as an indication of localized infection from necrotic enteritis. cecal tonsils Zn/Cu-SOD-1 expression on day 21 was statistically significant between treatments (*$P < 0.05$). Zinc glycinate at 140 mg/kg reduced Zn/Cu-SOD-1 expression on day 21 in NE-infected birds. Spleen IFN-γ (*$P < 0.05$) and TNF-α (*$P < 0.01$) expressions on day 21 were statistically significant between treatment but the expressions were not different in NE-infected birds.

DISCUSSION

Zinc is co-factors in immunity such as the antioxidant enzyme, Zn/Cu superoxide dismutase, and thymulin, which regulates T cell proliferation. We previously demonstrated that zinc modulates immunity through pro- and anti-inflammatory cytokines in healthy chickens (manuscript submitted). Currently, zinc is fed at 100 mg/kg in poultry diets. We conducted this study to elucidate the effect of zinc at the current recommendation and beyond on immunity during an infection. We reduced necrotic infection as evaluated by mortality and gross lesions by supplementing zinc glycinate. However, this high concentration of zinc did not affect immune responses as evaluated by cytokines and T helper cells population. Most notably, Zn/Cu-SOD gene expression was decreased at 140 mg/kg of zinc glycinate in necrotic enteritis. Because of this, we believe that zinc glycinate alleviated the infection through immune pathways independent of cellular immunity. In future studies, we will investigate the effects of zinc glycinate on innate immunity in chickens.

ACKNOWNLEDGEMENTS

Zinc glycinate was donated by Pancosma SA (Geneva, CH). The help and suggestions provided by Brandi Sparling, Mohamad Mortada, Gabriele Akerele, Keila Aceveda, Bailey Lester, Jarred H. Oxford, Alberta Lorraine Fuller, Charles Hofacre, Bret Lumpkins, Greg Mathis, and Julie Nelson are appreciated.

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Figure 1. Lesion scores (top) and mortality (bottom) of the birds on day 21.

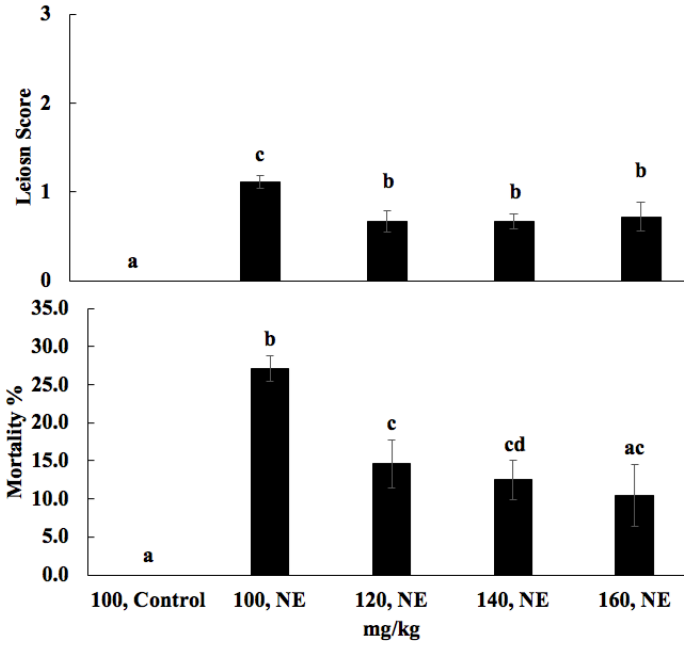
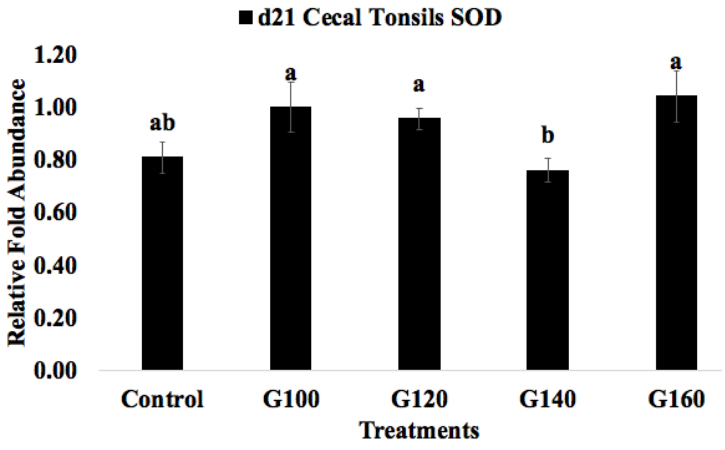


Figure 1. Day 21 cecal tonsils Zn/Cu-superoxide dismutase gene expressions. *P < 0.05.



REOVIRUS VARIANTS AND LAMENESS IN ONTARIO BROILERS

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SUMMARY

Avian reoviruses are non-enveloped containing double stranded degmented RNA. They are ubiquitous and are often considered non-pathogenic. However, some avian reoviruses have been associated with diseases such as viral arthritis/tenosynovitis, respiratory disease, enteric disease, immunosuppression and malabsorption syndrome. Reoviruses that infect joints primarily cause tendons inflammation and rupture, but also pericarditis, myocarditis, hydropericardium and uneven growth. Incubation period and disease production depends on virus pathotype, route of exposure, age and immune status. Reovirus interaction with other infectious agents can impact the nature and severity of reovirus-associated disease.

Before 2012 reovirus-associated leg issues were just sporadic disease in Ontario broilers. However, in 2012-2013 and 2017-2018 outbreaks of reovirus-associated lameness resulted in significant bird suffering and serious economic losses as some flocks had to be depopulated. Clinically, birds exhibited lameness that ranged from difficulty when walking to inability to walk due to leg deformities, including splayed legs and swollen hocks. Affected flocks were of variable ages, but flocks that were infected at a younger age, presented with a more severe clinical picture, with higher mortality and cull rates. Histologic lesions included non-suppurative tenosynovitis and epicarditis typically composed of lymphocytes and plasma cells forming well developed lymphoid nodules. Genotyping results determined that several "variant" strains of avian reovirus were involved. Clinical presentation and intensity of histopathologic lesions were often strain dependent, and cull rates ranged from 2-3% to 50%. In the spring of 2019, vaccination of Ontario broiler breeders with an autogenous reovirus vaccine was instituted and had a positive impact on broiler leg health in Ontario.

LINKING REMOTE SENSING OF WATERFOWL TO ULTRA-FILTRATION AND WHOLE SEGMENT PCR OF TARGETED WETLANDS FOR AVIAN INFLUENZA DETECTION IN THE CENTRAL VALLEY OF CALIFORNIA

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SUMMARY

Migratory waterfowl, including geese and ducks, are indicated as the primary reservoir of avian influenza viruses (AIv) which can be subsequently spread to commercial poultry. The US Department of Agriculture's (USDA) surveillance efforts of waterfowl for AIv have been largely discontinued in the contiguous United States. Consequently, the use of technologies to identify areas of high waterfowl density and detect the presence of AIv in habitat such as wetlands has become imperative. Here we identified two high waterfowl density areas in California using processed NEXt generation RADar (NEXRAD) and collected water samples to test the efficacy of two tangential flow ultrafiltration methods and two nucleic acid based AIv detection assays. Whole-segment M-RTPCR yielded more positive samples than standard M-segment qPCR methods (50.0% vs 2.6%, $p<0.0001$). We determined that this difference in positivity was due to mismatches in published primers to our samples and that these mismatches would result in failing to detect in the vast majority of currently sequenced AIv genomes in public databases. The whole segment sequences were subsequently used to provide subtype and potential host information of the AIv environmental reservoir. There was no statistically significant difference in sequencing reads recovered from the RexeedTM filtration compared to the unfiltered surface water. This overall approach combining remote sensing, filtration, and sequencing provides a novel and potentially more effective, surveillance approach for AIv. The full length article will be published in Transboundary and Emerging Diseases.

INTRODUCTION

Due to the presence of influenza A viruses in waterfowl which can be excreted from fecal/oral routes into the environment (1), surveillance of AIv's in waterfowl habitat play a vital role in the transmission of AIv (2-5). While current national surveillance of AIv in commercial and backyard poultry is rather extensive temporally and spatially, the source waterfowl population, remains relatively under-surveilled. Combining a more sensitive environmental AIv sampling technique with more targeted sampling of wetlands where waterfowl occur in high densities could lead to greater efficiency and effectiveness of surveillance. The national network of weather surveillance radars (i.e., NEXRAD) is a remote sensing tool that offers the ability to rapidly identify the location of waterfowl in close proximity to commercial poultry. NEXRAD is proven to comprehensively and accurately quantify aggregate waterfowl biomass and distribution near the ground using an instantaneous measure of radar reflectivity at the onset of highly-synchronized flights of wintering waterfowl departing their daytime roosting locations as they fly to nighttime feeding locations (6). Thus, using NEXRAD to identify high density waterfowl locations to target efficient testing for the presence of AIV and communicating that information seamlessly to commercial poultry farmers and relevant state and federal animal health authorities is essential toward preventing future outbreaks of AIv in commercial poultry.

The goal of this study was to develop a sensitive and targeted detection method for AIv in wetlands with high waterfowl density as a foundational step to improving environmental surveillance. To reach this goal we:

1) Used weather surveillance radar observations to identify two wetlands with high waterfowl density.
\2) Tested two filtration methods to concentrate AIv in water samples from those wetlands.

3) Tested two nucleic acid detection methods (e.g. multi-segment RTPCR vs M-segment qPCR).
4) Provided sequence information detailing the molecular viral ecology of AIV in sampled wetlands.

MATERIALS AND METHODS

Historic (i.e. 2014) radar reflectivity from three NEXt generation RADar (NEXRAD) stations (KBBX, KDAX and KHNX) in the Central Valley of California were used to identify wetlands with high waterfowl density and distribution (Figure 1). NEXRAD is a remote sensing tool proven to quantify waterfowl density and distribution near the ground using an instantaneous measure of radar reflectivity at the onset of highly-synchronized flights of waterfowl departing their daytime roosting locations as they fly to nighttime feeding locations (6).

Water samples were collected between June 2018 and November 2018. During each sampling interval five locations were chosen randomly with GPS marking. Measurements of pH, temperature, and salinity were recorded with the YSI Professional Plus sensor at each of the five locations within a wetland. 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 (7). A total of five 45 mL sediment samples were collected at each wetland interval to compare the presence and persistence of AIv in sediment to water samples (8). Water samples were stored on ice and taken back to the lab for same day filtration.

The rationale of ultrafiltration is to concentrate large volumes of wetland water for a more representative sample that is indicative of overall AIv presence in the environment. 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 (9). Viral particles were retained by molecular weight cutoffs and concentrated in the retentate while molecules smaller than the filter's pore size flowed through the membrane (10).

RNA from water and soil samples were extracted using the QIAamp Viral RNA Mini Kit (QIAgen, Valencia CA) on a QIAcube and the PowerViral Environmental DNA/RNA Isolation kit (QIAgen, Valencia CA), respectively. Following extraction, two methods were used to detect the presence of AIv in samples: reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) and whole segment amplification followed by sequencing. With respect to the RT-qPCR, a conserved ~100bp fragment of the matrix protein is amplified according to Spackman et al. (12). Whole-segment amplification was attempted using multi-segment RT-PCR (M-RTPCR) (13). 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, M-RTPCR amplifies entire gene segments if they are present in the sample. We conducted gel electrophoresis to assess genome segment amplification and completed multiplexed sequencing of amplicons using the Oxford Nanopore MinION sequencer (Oxford Nanopore Technologies, UK). The primers presented overhangs with 5' 22bp barcodes (shared among all samples) and 3' 8bp barcodes that were unique for each sample. The MinION sequenced single DNA molecules and allowed for the recovery of entire influenza genome segments (14).

To evaluate the efficiency of M protein RT-qPCR procedure we used herein (6), we conducted two computational analyses on all fully sequenced avian influenza genomes in IRD as of August 12, 2019. First, we searched for exact matches for primers and probes and used this as an initial indicator of the probability of a successful assay. Second, to conduct a more realistic analysis of assay success (as PCR often tolerates primer mismatches) we conducted a thermodynamic simulation of the TaqMan assay using ThermonucleotideBLAST (15) which outputs whether an amplicon was generated (together with its length and sequence) for each sequence under the specified conditions. To verify discrepancies between positive samples detected with qPCR vs. M-RTPCR/sequencing, we employed the same two analyses to examine results for our field-collected samples.

RESULTS AND DISCUSSION

The results of this study provide evidence for the feasibility of an AIv monitoring approach that combines various novel technologies with respect to AIv surveillance. Twith high waterfowl distribution and density with various wetland water sampling techniques (filtered vs. un-filtered) and PCR based approaches (matrix RT-qPCR vs whole-segment PCR).

Initial tests suggested sample concentration on AIv sequence recovery was not different from unfiltered surface water. However, our data set included an unfiltered sample that yielded over an order of magnitude more sequencing reads than any other sample. Excluding this outlier sample, filtration of environmental water samples by Asahi Kasei RexeedTM 25s yielded more sequence data, on

average, compared to unfiltered surface water samples, presumably by retaining more viral particles. However, this difference was not statistically significant, likely due to the small number of samples. The outlier sample possibly represents a "jackpot" scenario of sampling an area of high AIv. More samples are needed to definitively establish the effect of filtration on viral recovery over multiple seasons. The two filters likely performed differently due to the molecular weight cut off (MWCO) and surface area/flow rate differences of the two filters. The larger pore size and overall design of the Asahi Kasei RexeedTM 25s was proven to be better fit at retaining viral particles.

While filtration did not improve read recovery, the M-RTPCR and long-read sequencing approach yielded sensitive detection from unfiltered surface water samples that were missed using the standard M-segment RT-qPCR approach. Regardless of filtration method, M-segment RT-qPCR yielded one positive sample (out of 36), versus 19 using sequencing. Thus, this sequencing approach could be a powerful alternative to RT-qPCR, whether filtration is used or not. However, it is important to note that these methods have different goals by design. The M-segment RT-qPCR detects a 100bp fragment of a particular influenza segment. The MinION sequencing platform, as used here, recovers full influenza genome segments relying on the conservation of packaging regions (Zhou et al. 2009). The results herein together with the known molecular instability of RNA in solution, potentially suggest that we were recovering RNA directly from the capsids, which would protect RNA from damage or degradation prior to RNA extraction. It is extremely unlikely to obtain sequencing reads >2.2kpbs (n=80) of RNA with even low levels of RNA degradation. This is in contrast to the RT-qPCR method, which by design only amplifies a small conserved segment of RNA,that may or may not be representative of intact viral particles present. Further testing including electron microscopy and virus isolation is needed to determine to what extent sequences are in fact derived from intact viral particles, and whether those particles retain infectivity.

CONCLUSION

The needs for sensitive methods of AIv detection are in high demand. This sequence data provides information on the genetic diversity and composition of influenza viruses in the water column and sediment. Sequence data could be used to determine influenza virus subtypes present, infer time-space influenza virus sequence dynamics, and relate these sequence patterns to larger scale, ongoing influenza virus dynamics using independent surveillance data. Furthermore, assessing the strength of the link between remotely-sensed waterfowl density and viral load in the environment is needed. This could be done by testing spatio-temporal correlations of AI prevalence in environmental samples with concurrent radar- observed bird density at multiple sampling locations repeated over short time intervals (e.g., bi-weekly or monthly). Such analyses will enhance our knowledge on the nature of the AIv waterfowl reservoir and allow us to couple remotely sensed patterns of bird movements to the risk of specific AIv groups for a strong surveillance tool.

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SEROLOGICAL SURVEILLANCE OF INFECTIOUS BURSAL DISEASE VIRUS IN WESTERN CANADA

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ABSTRACT

Variant infectious bursal disease (vIBDV) is an economically important immunosuppressive disease in broiler chickens. These vIBDVs can be classified as sub-clinical as they lead to secondary infections, reduced weight gain and overall poor flock performance. Therefore, the objective of this study was to evaluate the prevalence of IBDV in western Canada. Sera was collected from broiler flocks at processing from British Columbia (n=78), Alberta (n=68) and Saskatchewan (n=52). The geometric mean titers from each flock were determined using a commercial ELISA. Broiler flocks were then categorized as IBDV positive or negative. Production information such as condemnations, mortality and average live weight were collected. The results of this study revealed 45% of flocks tested in British Columbia were positive for IBDV, 6% in Alberta and 48% positive in Saskatchewan. Condemnation rates in IBDV positive flocks were higher than in IBDV negative flocks. Further research is needed to identify the strain of vIBDV responsible in positive flocks and factors leading to the low incidence of positive flocks in Alberta.

INTRODUCTION

Belonging to the family *Birnaviridae*, vIBDV causes long lasting immunosuppression in chickens when infected less than 3 weeks of age (3). In Canada, approximately 60% of IBD cases were linked to vIBDV (4,5), leading to increased condemnations (1) and production losses (6).

MATERIALS AND METHODS

Sample collection. A total of 198 broiler flocks from British Columbia (n=78), Alberta (n=68) and Saskatchewan (n=52) between 2011 and 2013 were included in this study. At the time of processing, sera was collected from 20 broilers per flock.

Serologic study. The geometric mean (GM) titers from each flock were determined using a commercial enzyme linked immunosorbent assay (ELISA) kit at the Animal Health Laboratory, University of Guelph. Flocks were then categorized as IBDV negative (GM <999) or positive (GM >1000).

Data collection. Production information such as condemnations, mortality and average live weight were collected for each flock.

RESULTS

Serologic study. Of the flocks tested, 45% were positive in British Columbia and 485 were positive in Saskatchewan while only 6% were positive in Alberta. The IBDV GM were 1785, 260, and 2122 in British Columbia, Alberta, and Saskatchewan respectively.

Economic impact. Condemnation rates in were higher in flocks positive serologically with IBDV. The average total percent condemnations in British Columbia were 1.38% and in Saskatchewan 1.26% while in Alberta were 0.73%.

DISCUSSION

In Canada, vIBDV is the most prevalent IBDV circulating (5). The virus targets the bursa of Fabricius, and the resulting loss of lymphocytes leads to severe immunosuppression (3). Not only does vIBDV cause direct losses to the broiler industry but also indirectly due to secondary infections. Previous we reported 43% of broiler farms in Saskatchewan were positive for IBDV in 2009 (6) and also earlier determined an association between positive IBDV titers and hepatic condemnations in the broiler industry in Saskatchewan (1). In this study, we found higher overall condemnations in flocks with increased IBDV titers. In a previous follow-up study, 92% of farms maintained IBDV infection between 2007 to 2011 (6). Our results from this study are consistent with previous findings, as IBDV is resistant to many disinfectants and is difficult to remove from a poultry barn (2,4). In Saskatchewan alone, it was estimated 3.9

million kilograms/year of broiler meat is lost with a wholesale market value of approximately 13 million CAD (6). The results from this study indicate IBDV is prevalent in British Columbia and Saskatchewan but not in Alberta. Further research is needed to identify the strain of vIBDV responsible in positive flocks and factors leading to the low incidence of positive flocks in Alberta.

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INFECTIOUS BRONCHITIS VIRUS SURVEILLANCE IN BROILERS BEFORE AND AFTER LIVE VACCINATION

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ABSTRACT

The purpose of this study was to determine the effects of a vaccine intervention in response to an increased condemnation rate, due to airsacculitis of broiler chickens in California. Necropsy reports of affected birds were evaluated for symptoms of IBV infection and past exposure by performing PCR and ELISA on samples collected before and after the vaccine was implemented. Additionally, disease index was compared between different seasons throughout the year to account for seasonal effects on IBV prevalence. Overall, the effects of the vaccine were largely masked by seasonal effects. We are currently collecting data on birds vaccinated and raised during the winter months in order to correlate it with the data collected from vaccinated birds during the summer season.

INTRODUCTION

The gamma coronavirus responsible for infectious bronchitis has a large genetic diversity that is difficult to protect against with vaccination. In California, like in most of the world, infectious bronchitis is endemic, with several variant strains isolated from farms with affected birds (1). The virus causes upper respiratory signs, posing a threat to bird welfare and economic losses for the farmer; surveillance of the IB virus and birds' response to vaccination are important to assess the effectiveness of IBV vaccines in the field.

MATERIALS AND METHODS

In this study, a live Massachusetts + Conn 46 strain vaccine was administered to broilers at day of age, and disease surveillance was performed on necropsy submissions from both before and after the vaccination protocol was initiated. Data was collected through IBV RT-PCR, virus isolation, ELISA titers, and disease index at the processing plant. The surveillance started in December 2018 and is still ongoing.

RESULTS AND DISCUSSION

Overall, the average IBV GMT titer value decreased from pre-vaccination to post-vaccination state (Samples were collected in average at 30 days of age). However, through T-test analysis, only 5 sites showed a significant decrease in titer value, while 4 sites had a significant increase ($p<0.05$). The isolates molecularly characterized before vaccination were the CA 3099, Mass41, and Conn 46 strains, while after vaccination only Conn 46 was isolated. This Conn 46 strain matched with the strain of the live vaccine used. Before vaccination, 10 IBV viruses were isolated, while only 2 were isolated after vaccination, as summarized in Table 1. Even though, these results show a predominance of vaccine strains in the field it also coincided with the summer months, reason why we are continuing the surveillance in vaccinated birds during the winter months.

The change in GMT and PCR results is summarized in Figure 1, along with the average age and number of birds sampled both before and after vaccination.

The disease index, measured at processing was highest in the rainy months, late fall to winter. The prevalence dropped throughout spring and summer to reach its lowest rate at the beginning of fall in 2019. The significance of disease index as it relates to season will be further discussed, as well as its effect on perceived versus true vaccine effectiveness.

CONCLUSION

In conclusion, this surveillance project should be repeated with vaccination in the winter months to more accurately assess the effectiveness of the IB vaccine in the field. The rainy season predisposes birds to IBV infections. The decrease in disease incidence may be due to vaccination or to a reduction of the virus in the environment that would have occurred naturally with the drier season. The strains of IBV isolated and characterized should be recorded and updated on a yearly basis in order to survey virus migration and

transformation over time. The best vaccine protocol (use or not use) may change as the viral variation and prevalence fluctuates seasonally and annually. More interpretations and conclusions will be discussed further as more data become available.

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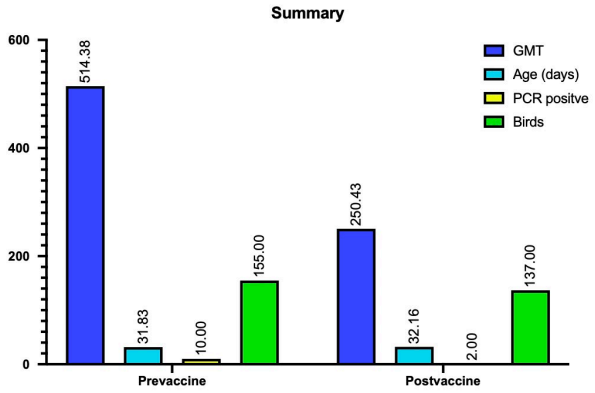
Table 1. Proportion of each strain isolated before and after vaccination, by PCR.

	CA 3099	MDL 15	Conn 46
Pre-vaccine	3/10	6/10	1/10
Post-vaccine	0	0	2/2

Figure 1. The average titer (GMT) decreased over time from 514.38 to 250.43. The number of birds positive for IBV on PCR decreased from 10 to 2. The age and sample size of the pre- and post-vaccination groups was comparable.



HOUSING, MANAGEMENT, WELFARE, AND DISEASE – IMPACTS AND MITIGATION FOR TODAY'S LAYER PULLET AND LAYING HEN

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INTRODUCTION

Laying hen welfare occupies the intersection of hen health and behavior, human health and behavior, equipment and the environment. The role of the veterinarian is to identify risks, prevent problems and preserve health of humans and animals. If the consumer wants "green eggs and ham," it is our role to find a way to provide it while preserve hen well-being and food safety. Solving problems in the field requires drawing on a mixture of the scientific literature, field observations, and classical animal training techniques.

NESTING BEHAVIOR

The economic goal of cage free production is to produce a clean, saleable egg. The majority of hens have a preference for laying in nests and will exhibit restlessness and searching behavior in a laboratory setting around the time of oviposition. However, an economically significant portion of hens lack the same drive. Zupan *et al*. (25) found that 70% of hens lay consistently in the nest with the remainder laying in litter trays. This behavior trend persists across genetics over time. Cooper and Appleby (6) found the same 70/30 split on nest use and it mirrors field results in untrained flocks.

Farmers use nest design, pullet house design, training, and stocking densities to improve the percentage of hens laying in the nest. Bari *et al*. (2) confirmed that pullets grown in environments with weekly changes lay a higher percentage of nest eggs than pullets reared in more controlled environments.

Different strains have different nesting behavior and farmers need to pick the strain that matches their needs. Villanueva *et al* (24) found that Hyline and Bovan browns spent more time in the nest in the morning and laid more nest eggs at the time. In contrast, W-36 and Dekalb white strains dominated the nests and nesting behavior in the midday. Matching nest design and management techniques to strain improves economic outcomes.

Numerous investigators have examined the preferences of different strains in terms of nest design, substrate, positon, size, and lighting (1, 22, 24). These findings are useful in choosing housing systems, addressing problems with nest use, and when making decisions about remodeling equipment.

FEATHER PECKING

Daigle *et a*l. (7) established that although aggressive feather pecking behavior is persistent in around 5% of hens while nearly 8% live as constant victims of their feather pecking neighbors, over 50% do not consistently peck or receive pecks. Results such as these indicate that inventions could be successful in reducing pecking in aggressive flocks. These results also help explain some of the challenges of larger flock and enclosure sizes where those persistent 5% have access to a larger portion or all of the flock.

PERCHING

Perching reduces pecking and aggression by providing levels for hierarchy expression. It encourages vertical use of housing systems when provided to pullets and may influence fearful behavior. Proper perch design includes attention to bird height and bird foot size and is crucial to achieving these benefits (3, 23). Perching should be adequate in linear space to bird numbers and bird size to see the greatest benefits (19).

Access to perching is also consistently associated with keel bone malformations and fractures (1). It is unclear if these are solely due to the damaging effect of impacting and entangling in the equipment or if the act of perching contributes to bone malformation. Specific interventions in terms of housing design and genetics may mitigate some of these problems (10). Hens with keel bone damage exhibit reduce behavior expression and reduced production (18).

SPACE

Floor space is one component. Care must be taken to balance feeder space, water access, and to examine if calculable space is actually usable space (21). Mench and Blatchford (15) examined how hens used square footage to perform various limb extension

behaviors allowing farmers calculate how much space to allow for expression without interference with pen mates.

Population size as it impacts social rank is another, related factor. When faced with limited resources, hens of lower social rank preform less dust bathing and spend more time hiding in the nests (20). Even nest searching behavior is influenced by the presence of dominate hens. Submissive hens will take alternate routes or shorten their nest searching time (8). Chen and Bao (5) found that group size impacted all most all behavior when they attempted to study the impact of perch color, which had little correlation on behavior.

Diurnal rhythms impact bird utilization of the system throughout the day. Allotted space that may be adequate during some times, might be scarce at others resulting in adverse bird behavior (4).

PLUMAGE

Feather cover can be impacted by ectoparasites, equipment wear, feather pecking, nutrition, feed constraints, gut health, and high production (9). Care must be taken to establish the cause and extent of feathering problems before embarking on a solution. Further, care must be taken when interpreting literature related to plumage and its applicability to the specific field conditions. Some housing systems allow for the use of novel methods of ectoparasite control such as dust bath usage (14, 17).

REMODELING

Lentfer *et al*. (12) found that placement of nipple drinkers relative to nest location impacts hen stationary and locomotion behaviors. Optimal placement leads to more even distribution of the hens throughout the systems.

Farmers that examine the original purpose of welfare program recommendations and develop housing systems with bird use and bird preference in mind often find their remodels more cost effective with lower reported bird behavior issues (3, 16).

Furnished cages consistently perform well in studies examining hen behavior and production (11, 13).

CONCLUSIONS

Newer housing systems and increased consumer attention to hen behavior creates a need to address challenges in novel ways. Access to manure and dust predispose hens to infectious disease. New vaccines and improved housing can mitigate these risks. Increased labor quality and attention to detail is crucial for producing a high-quality, safe egg under our modern constraints. Controlled research, classical disease knowledge, and hen observation help veterinarians manage outcomes in the increasing complex ecosystem that makes up the modern layer barn.

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GENOMIC ANALYSIS OF *AVIBACTERIUM PARAGALLINARUM* FROM 2019 INFECTIOUS CORYZA CASES IN THE UNITED STATES

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SUMMARY

Since February of 2019 infectious coryza suspect cases with symptoms including decreased consumption of feed and water, and swollen heads and wattles with catarrhal discharge were submitted from commercial layer farms in Pennsylvania and nearby states. *Avibacterium paragallinarum* was successfully isolated and identified from bacterial cultures from infra-orbital sinus swabs. In addition to classical phenotyping analysis of the isolates, whole genome sequencing (WGS) was performed to identify genetic relationships between the isolates. The outer membrane haemagglutinin gene (hmtP210) showed a high degree of similarity amongst all regional isolates and clustered with serotype C reference serovars. However, pan-genome analysis of core genes showed the distinct clusters of the isolates and analysis of the accessory genes further separated them by states.

INTRODUCTION

Infectious coryza is an acute avian respiratory disease caused by *Avibacterium paragallinarum* and the symptoms include decreased feed and water consumption, swollen heads and increased nasal discharge. The disease has significant economic impact in layer operations causing significant egg production drop and poor growth performance. It also has an impact on condemnation rates increase in the broiler industry (1). Ceva Scientific Support and Investigation Unit (SSIU) have received clinical samples during the 2019 outbreak of infectious coryza in Pennsylvania and the surrounding regions. The goal of this study was to characterize *Avibacterium paragallinarum* isolates to help in understanding the epidemiology of the current outbreak.

MATERIALS AND METHODS

Bacterial culture. During PA coryza outbreak, we have received nine infectious coryza suspect cases consisting of 132 samples of whole heads or swabs representing 24 layer flocks across four states: Delaware = 1, Maryland = 1, Pennsylvania = 5, and Virginia = 2. Upon reception of the whole head samples, the beaks were removed for nasal swab collection. All samples were plated for isolation on 5% sheep blood agar (Hardy Diagnostics, Santa Maria, CA) with a staph nurse and chocolate agar (Remel Inc., San Diego, CA). Cultures were incubated for up to three days and isolates confirmed as *Avibacterium paragallinarum* were stored for further analysis. Bacterial cultures were identified using the appropriate API® identification system (BioMerieux, France).

Whole genome sequencing. DNA was extracted from all isolates and sequenced on Ion Torrent S5 sequencer. Sample raw reads were trimmed and curated with FASTP (2). Trimmed data was assembled *de novo* with SPADES (3) and the contigs were aligned to *Avibacterium paragallinarum* reference genome (CP034110.1) using RAGOO (4). Aligned genomes were verified to be *Avibacterium paragallinarum* with KRAKEN2 (5). Aligned samples genomes were annotated with PROKKA (6). Annotated genomes were scanned for pan-genome with ROARY (7) to perform Pan-Genome analysis. All genes within a sampling of isolates were assessed and divided into core and accessory genes (shell, and cloud) groups (8). Core genes are those present in greater than 99% of all genomes sampled and are usually required for function. These genes are present in all genomes of a given clade. Shell genes are present in 15% - 94%, and cloud genes are present in less than 15% of all genomes sampled. These accessory genes are acquired but may not be necessary for function (9). Pan-genome tree was built using FASTTREE (10). *In silico* analysis of the haemagglutinin gene hmtp210 was performed using Geneious Prime® 2019.2.1. The haemagglutinin sequence was extracted from *Avibacterium paragallinarum* reference genome (CP034110) and mapped against all curated genomes. All haemagglutinin sequences were reduced to roughly 1.7kb using the primers 5-1 F: GATGGCACAATTACATTTACA and 5-1 R: ACCTTGAGTGCTAGATGCTGTAGGTGC

(11,12). Serotype reference sequences were used to attempt to serotype the isolates13.

RESULTS AND DISCUSSION

Bacterial culture. In addition to Avibacterium paragallinarum, most of the samples yielded growth of other pathogenic bacteria such as *Ornithobacterium rhinotracheale*, *Gallibacterium anatis* (both biovars), *Erysipelothrix rhusiopathiae*, *Bordetella* spp., enterococci, and *E. coli* from moderate to heavy amounts. From the total of 132 samples, we recovered 46 *Avibacterium paragallinarum* isolates in light to moderate amounts. Clinical presentations and bacterial isolation confirmed infectious coryza cases; however, it should be noted that the other pathogenic bacteria isolated together from the samples may have played an important role in pathogenicity of the coryza cases and its epidemiology. *Avibacterium paragallinarum* is a catalase negative, oxidase positive gram negative rod. It is a fastidious bacterium, requiring NAD for growth although NAD independent strains have been described (1). Among all isolates, biochemical tests using API® NH strips varied only in the presence or absence of the enzyme gamma glutamyl transferase.

Whole genome sequencing. Forty-six complete whole genome sequences were obtained and Pan-Genome analysis was conducted. There were 4284 genes in total, and 1396 core genes were present in all 46 isolates. Figure 1 show the Pan-Genome tree built from a nucleotide alignment of all 1396 shared core genes. The relationship among core genes shows that isolates from Virginia are in a different clade than those from the other states. Analysis of 35 isolates using accessory genes is shown in Figure 2. The alignment of accessory genes show diversity between the isolates and clustered them mostly by state location.

Additionally, we attempted to identify the serotypes of the *Avibacterium paragallinarum* isolates utilizing haemagglutinin gene. The widely accepted method of serotyping is the Kume method (14), and this classical serotyping method types *Avibacterium paragallinarum* into 9 serotypes (A-1, A-2, A-3. A-4, B-1, C-1, C-2, C-3 and C-4). However, classical serotyping method is difficult and reagents are hard to acquire. There has been an attempt to correlate serotyping of *Avibacterium paragallinarum* with multiplex PCR amplification of haemagglutinin hmtP210 hypervariable region (12). In this study, we trimmed region 2 of hmtP210 gene utilizing the primer set (⊿ 5-1) (12) and created a phylogenetic tree in an attempt to find a correlation between serotype and hmtP210 gene sequence (Figure 3). Reference sequences obtained from the National Center for Biotechnical Information (NCBI) of known serotypes, used by Wang, H. *et al*. (13), were included in the analysis. As shown in Figure 3, haemagglutinin gene region 2 analysis could not clearly separate all the reference sequences with known serotypes accordingly. Serotype A was separated into two major cluster with some minor outliers. Also serotype C (C1~C4) shows one major cluster with minor mixed in other serotype cluster. However, the isolates from this study all clustered with the majority of serotype C.

CONCLUSION

There are a number of methods used to analyze a group of isolates with the goal of understanding their epidemiology, pathogenicity and diversity. Phenotypes can distinguish isolates biochemically or by serotype. Molecular PCR methods can detect the presence of specific virulence genes further characterizing an isolate. WGS can provide additional information to aid in understanding among this diversity of isolates. In this study, we focused on pan-genomic analysis, instead of focusing on limited virulence factors, to understand the diversity of the *Avibacterium paragallinarum* isolates we isolated in multiple locations. The data showed that the isolates were relatively contained within the state of origin. Haemagglutinin gene analysis for serotyping *Avibacterium paragallinarum* for this group of isolates gave an indication of its association with serotype C. While this method may be useful in determination of A, B or C, complete serotyping to the level of Kume methodology is still allusive.

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Figure 1. Pan-Genome tree of *Avibacterium paragallinarum* core genes.

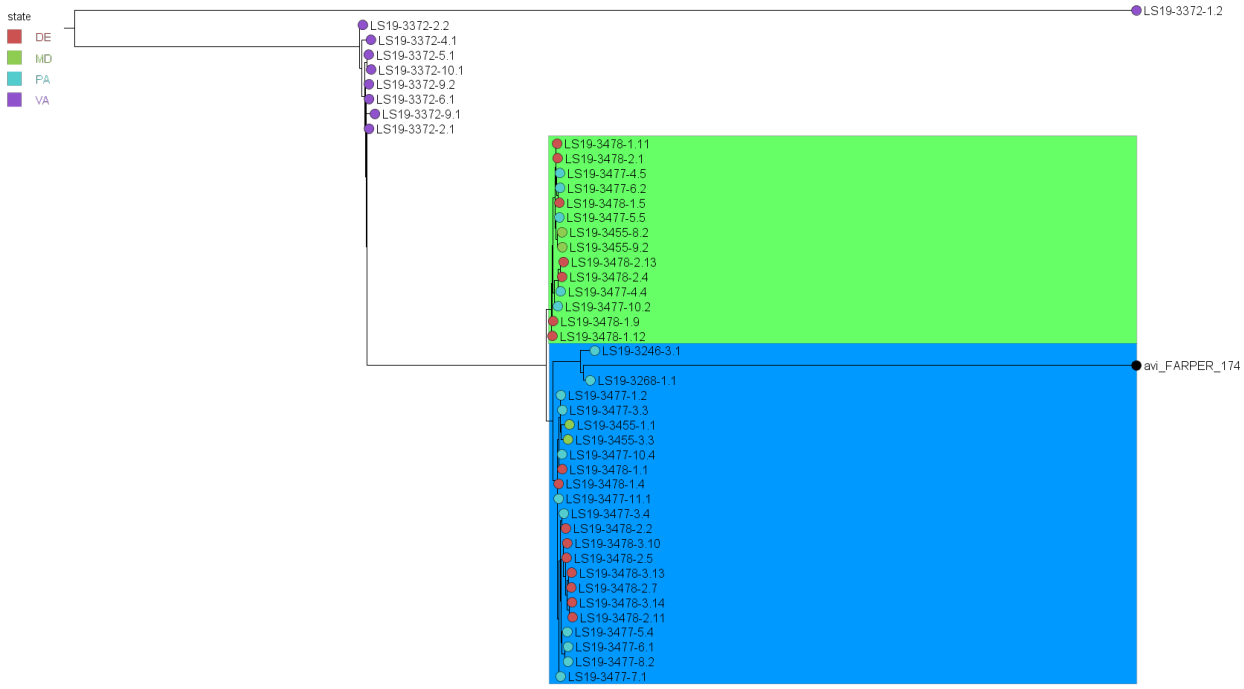
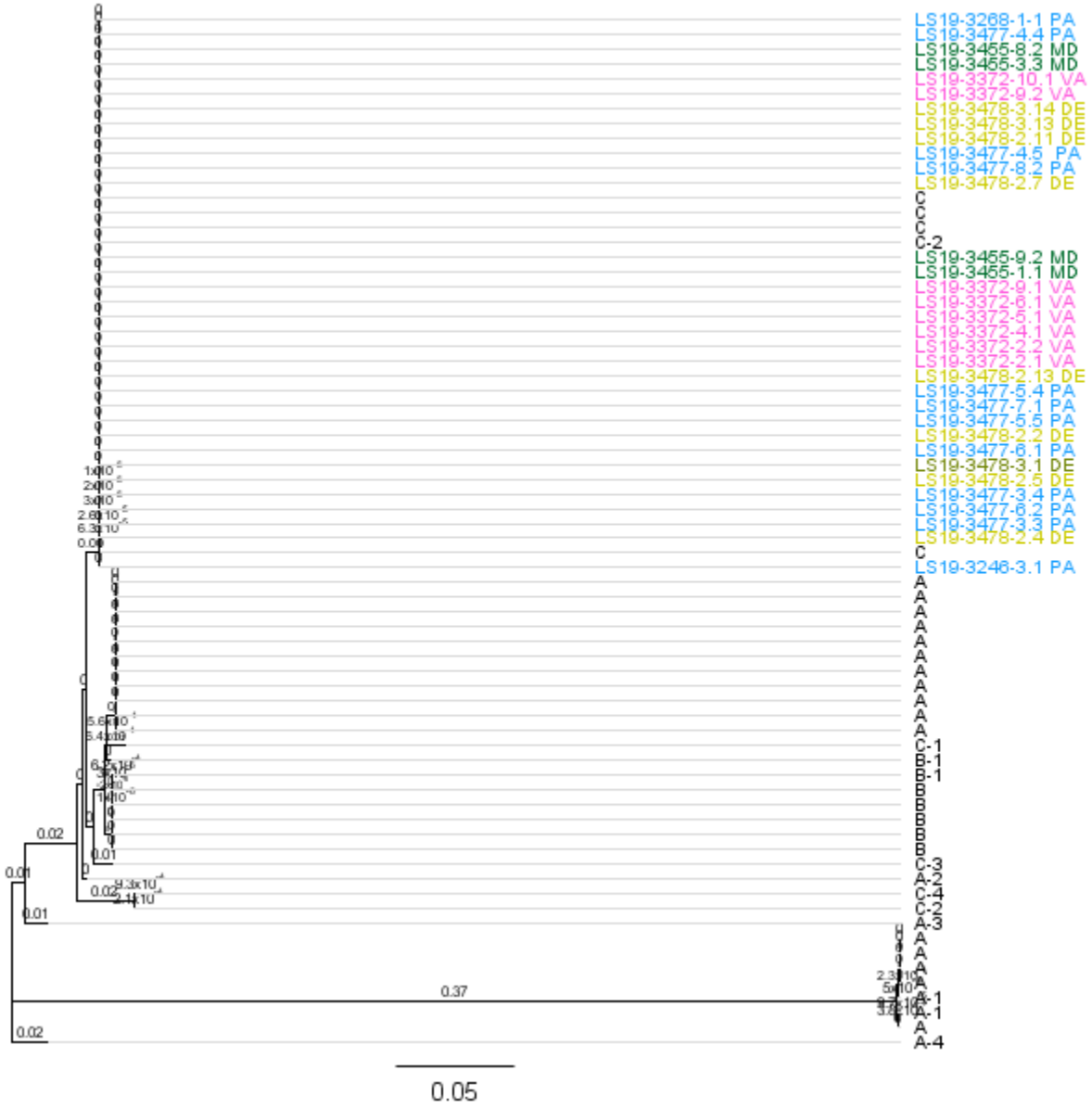


Figure 2. Pan-Genome tree of *Avibacterium paragallinarum* accessory genes.

Figure 3. Haemagglutinin (HA) protein hmtP210 gene comparison.



COMPATIBILITY OF A RECOMBINANT HVT-ND VACCINE WITH BURSAPLEX TO PROVIDE PROTECTION AGAINST VELOGENIC NDV AND VIRULENT CLASSIC IBDV CHALLENGE IN SPF BIRDS

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SUMMARY

A recombinant HVT-ND (Poulvac® Procerta™ HVT-ND) was developed as a bi-valent vaccine for protection against Newcastle disease (ND) and Marek's disease (MD). Studies were conducted and shown that HVT-ND and Poulvac Bursaplex® were compatible when administered together. Efficacy was observed with challenges of either an infectious bursal disease virus (IBDV) or Newcastle disease virus (NDV).

INTRODUCTION

ND is a highly contagious and fatal disease affecting all species of birds. MD is a common cause of condemnations and immune suppression in broilers. A recombinant HVT-ND bi-valent vaccine was developed. Due to vaccination routines in the field, studies were conducted to examine the compatibility of HVT-ND and Poulvac Bursaplex when administered together, followed by either an IBDV or NDV challenge.

MATERIALS AND METHODS

HVT-ND vaccine (Poulvac Procerta HVT-ND): HVT-ND is a recombinant viral vaccine. An NDV F gene expression cassette was inserted into the HVT genome. 0.05 mL/egg (in ovo), 0.2 mL/bird (subcutaneous injection)

Poulvac Bursaplex: Antigen (IBDV) Antibody Complex (AAC) vaccine

Challenge viruses: velogenic NDV Texas GB (USDA); virulent IBDV STC (USDA)

SPF birds: Leghorn CRL (Charles River Labs)

Allotment/ Randomization. All eggs to be allocated for the study came from a single incubator. At the time of transfer and in ovo vaccination (E18), eggs were distributed such that each area of the incubator is represented in each flat. Flats were individually numbered and randomized to treatment by the Biometrics representative. Treatments were then transferred to hatchers according to biosecurity constraints and the randomization.

All bird procedures were approved by the Institutional Animal Care and Use Committee.

RESULTS

For IBDV challenge study, 100% (30/30) of protection were observed for both in ovo and subcutaneous vaccinations. For NDV challenge study, 93% (37/40) and 95% (38/40) of protection were observed for in ovo and subcutaneous at hatch vaccination, respectively.

DISCUSSION/CONCLUSION

HVT-ND was experimentally combined with Bursaplex and shown to maintain its safety (based on mortality) and efficacy against a velogenic NDV challenge, as well as, a classical virulent IBDV challenge.

Serology results (% Signal Positive) of IBDV and NDV, measured by ProFlok IBDV Plus ELISA and NDV Plus ELISA kits, correlate with efficacy results for the two studies.

ACKNOWLEDGEMENTS

The authors wish to thank Zoetis ARS (Animal Research Service) for excellent animal work; and the HVT-ND project team and colleagues for help and discussions.

Table 1.

Treatment	Vaccine	Route	Challenge (D34)	% IBDV Efficacy (D38)		
				% Neg Bursal Lesions	% Negative Mortality	% Protection
T01	Diluent	*In ovo*	No	100 (30/30)	100 (30/30)	**NA (30/30)**
T02	CEF cells	*In ovo*	Yes	0 (0/24)	58 (14/24)	**0 (0/24)**
T03	HVT-ND + Bursaplex	*In ovo*	Yes	100 (30/30)	100 (30/30)	**100 (30/30)**
T04		Subcutaneous	Yes	100 (30/30)	100 (30/30)	**100 (30/30)**

Table 2.

Treatment	Vaccine	Route	Challenge (D28)	% NDV Efficacy (D42)		
				% Clinical Signs	% Live Birds	% Protection
T01	Diluent	*In ovo*	No	0 (0/40)	100 (40/40)	**NA (40/40)**
T02	CEF cells	*In ovo*	Yes	65 (26/40)	0 (0/40)	**0 (0/40)**
T03	HVT-ND + Bursaplex	*In ovo*	Yes	5 (2/40)	93 (37/40)	**93 (37/40)**
T04		Subcutaneous	Yes	2.5 (1/40)	95 (38/40)	**95 (38/40)**

ONSET OF IMMUNITY OF A RECOMBINANT HVT-ND AGAINST A VELOGENIC NDV CHALLENGE IN SPF BIRDS

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SUMMARY

A recombinant HVT-ND (Poulvac® Procerta™ HVT-ND) was developed as a bi-valent vaccine for protection against Newcastle disease (ND) and Marek's disease (MD). Two efficacy studies were conducted to explore the onset of immunity for this vaccine. In the first study, a velogenic NDV was used to challenge the birds, that were vaccinated either in ovo or subcutaneously, on Day 17, 18 and 19, followed by 14-day observation. In the second study, the birds, that were vaccinated either in ovo or subcutaneously, were challenged on Day 16 and 19. Above 93% protection were observed in both studies for Day 19 challenge with both in ovo and subcutaneous route of vaccination. In addition, dd PCR (droplet digital PCR) assay was developed to measure vaccine update by the birds.

INTRODUCTION

ND is a highly contagious and fatal disease affecting all species of birds. MD is a common cause of condemnations and immune suppression in broilers. A recombinant HVT-ND bi-valent vaccine was developed. Since maternal antibodies against NDV that present in most chicken start to wane within a few weeks after hatch, we want to understand the onset of immunity of this vaccine. Two independent efficacy studies were conducted.

MATERIALS AND METHODS

HVT-ND vaccine (Poulvac Procerta HVT-ND): HVT-ND is a recombinant viral vaccine. An NDV F gene expression cassette was inserted into the HVT genome. 0.05 mL/egg (in ovo), 0.2 mL/bird (subcutaneous injection).

Challenge viruses: velogenic NDV Texas GB (USDA)

SPF birds: Leghorn CRL (Charles River Labs)

Allotment/ Randomization. All eggs to be allocated for the study came from a single incubator. At the time of transfer and in ovo vaccination (E18), eggs were distributed such that each area of the incubator is represented in each flat. Flats were individually numbered and randomized to treatment by the Biometrics representative. Treatments were then transferred to hatchers according to biosecurity constraints and the randomization.

All bird procedures were approved by the Institutional Animal Care and Use Committee.

RESULTS

For the first study, 100% (40/40), 88% (35/40), 98% (39/40) of protection were observed for NDV challenges at Day 17, 18, 19, respectively, for in ovo vaccination. 75% (30/40), 88% (35/40), 93% (37/40) of protection were observed for subcutaneous vaccination on day of hatch. For the second study, 85% (37/40) and 93% (37/40) of protection were observed for Day 16 and Day 19 NDV challenge for in ovo vaccination. 70% (28/40) and 95% (38/40) of protection were observed for subcutaneous vaccination on day of hatch. In addition, ddPCR test results were shown to be more sensitive than the real-time qPCR results for measuring vaccine update,

DISCUSSION/CONCLUSION

93-98% efficacy was observed at Day 19/Day 33 post vaccination against a velogenic NDV challenge strain for both in ovo and subcutaneous routes of administration.

93-98% efficacy was observed at Day 19/Day 33 for both vaccination routes in two independent studies.

HVT DNA isolated from feather pulp was quantitated by both qPCR and ddPCR (digital droplet PCR) methods. ddPCR method is shown to be more sensitive to be used as a measure of vaccine update.

ACKNOWLEDGEMENTS

The authors wish to thank Zoetis ARS (Animal Research Service) for excellent animal work; and the HVT-ND project team and colleagues for help and discussions.

Table 1.

Trt	Vaccine	Route	Challenge (D17, D18, D19)	% NDV Efficacy		
				D31 (D17)	D32 (D18)	D33 (D19)
T01	Non-vaccinated	-	No	NA (40/40)	NA (40/40)	**NA (40/40)**
T02	Chall control	-	Yes	**0** (0/40)	**0** (0/40)	**0 (0/40)**
T03	HVT-ND	Subcutaneous	Yes	**75** (30/40)	**88** (35/40)	**93 (37/40)**
T04		*In ovo*	Yes	**100** (40/40)	**88** (35/40)	**98 (39/40)**

Table 2.

Trt	Vaccne	Route	Challenge (D16, D19)	% NDV Efficacy	
				D30 (D16)	D33 (D19)
T01	Non-vaccinated	-	Yes	**0** (0/40)	**0 (0/40)**
T02	HVT-ND	Subcutaneous	Yes	**70** (28/40)	**95 (38/40)**
T03		*In ovo*	*Yes*	**85** (34/40)	**93 (37/40)**

Table 3.

Trt	Vaccine	Route	FP* HVT qPCR % Positive		FP* HVT ddPCR % Positive	
			D8	D14	D8	D14
T01	Neg Control	-	-	-	-	-
T02	HVT-ND	Subcutaneous	0	64	83	100
T03		*In ovo*	46	57	83	100

*FP: feather pulp

DURATION OF IMMUNITY OF A RECOMBINANT HVT-ND AGAINST A VELOGENIC NDV CHALLENGE IN SPF BIRDS

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SUMMARY

A recombinant HVT-ND (Poulvac® Procerta™ HVT-ND) was developed as a bi-valent vaccine for protection against Newcastle disease (ND) and Marek's disease (MD). Duration of immunity was explored in this study. SPF birds that were vaccinated with HVT-ND were challenged with velogenic NDV on Day 63. 100% efficacy was observed for both *in ovo* and subcutaneous vaccination.

INTRODUCTION

Newcastle disease (ND) is a highly contagious and fatal disease affecting all species of birds. Marek's disease (MD) is a common cause of condemnations and immune suppression in broilers. A recombinant HVT-ND bi-valent vaccine was developed. This recombinant vaccine was tested for its duration of immunity by subjecting the HVT-ND vaccinated birds to NDV challenge on Day 63, followed by 14-day observation.

MATERIALS AND METHODS

HVT-ND vaccine (Poulvac Procerta HVT-ND): HVT-ND is a recombinant viral vaccine. An NDV F gene expression cassette was inserted into the HVT genome. 0.05 mL/egg (in ovo), 0.2 mL/bird (subcutaneous injection)

Challenge viruses: velogenic NDV Texas GB (USDA)

SPF birds: Leghorn CRL (Charles River Labs)

Allotment/ Randomization. All eggs to be allocated for the study came from a single incubator. At the time of transfer and in ovo vaccination (E18), eggs were distributed such that each area of the incubator is represented in each flat. Flats were individually numbered and randomized to treatment by the Biometrics representative. Treatments were then transferred to hatchers according to biosecurity constraints and the randomization.

All bird procedures were approved by the Institutional Animal Care and Use Committee.

RESULTS/CONCLUSIONS

100% efficacy was observed at Day 63 post vaccination against a velogenic NDV challenge strain for both in ovo and subcutaneous routes of administration.

Vaccine safety was observed. No clinical signs observed prior to NDV challenge for either *in ovo* or subcutaneous routes.

ACKNOWLEDGEMENTS

The authors wish to thank Zoetis ARS (Animal Research Service) for excellent animal work; and the HVT-ND project team and colleagues for help and discussions.

Table 1

Trt	Vaccine	Route	NDV Challenge (D63)	% NDV Efficacy		
				% Clinical Signs	% Mortality	% Protection
T01	Non-vaccinated	-	No	0 (0/30)	0 (0/30)	**NA (30/30)**
T02	Chall control	-	Yes	**80** (24/30)	**100** (30/30)	**0 (0/30)**
T03	HVT-ND	Subcutaneous	Yes	**0** (0/30)	**0** (0/30)	**100 (30/30)**
T04		*In ovo*	Yes	**0** (0/30)	**0** (0/30)	**100 (30/30)**

CONSTRUCTION AND EFFICACY OF A RECOMBINANT HVT-ND VACCINE AGAINST NDV AND MDV CHALLENGE IN SPF AND NDV CHALLENGE IN BROILER BIRDS

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SUMMARY

A recombinant HVT-ND (Poulvac® Procerta™ HVT-ND) was developed as a bi-valent vaccine for protection against Newcastle disease (ND) and Marek's disease (MD). Target antigen expression and recombinant stability were characterized for both *in vitro* and *in vivo*. Clinical studies were conducted to demonstrate efficacy against challenges of either velogenic NDV or virulent MDV in SPF leghorns. Efficacy of NDV was also demonstrated in broilers.

INTRODUCTION

ND is a highly contagious and fatal disease affecting all species of birds. MD is a common cause of condemnations and immune suppression in broilers. A recombinant HVT-ND bi-valent vaccine was developed. This recombinant vaccine was characterized for its target antigen expression, recombinant stability both *in vitro* and *in vivo*, NDV and MDV efficacy in SPF and NDV efficacy in broilers.

MATERIALS AND METHODS

HVT-ND vaccine (Poulvac Procerta HVT-ND): HVT-ND is a recombinant viral vaccine. An NDV F gene expression cassette was inserted into the HVT genome. 0.05 mL/egg (*in ovo*), 0.2 mL/bird (subcutaneous injection)

Challenge viruses: velogenic NDV Texas GB (USDA); virulent MDV (GA22)

SPF birds: Leghorn CRL (Charles River Labs)

Broiler birds: Commercial broiler eggs, straight run

Allotment/ Randomization. All eggs to be allocated for the study came from a single incubator. At the time of transfer and *in ovo* vaccination (E18), eggs were distributed such that each area of the incubator is represented in each flat. Flats were individually numbered and randomized to treatment by the Biometrics representative. Treatments were then transferred to hatchers according to biosecurity constraints and the randomization.

All bird procedures were approved by the Institutional Animal Care and Use Committee.

RESULTS/CONCLUSIONS

HVT-ND recombinant vaccine was constructed and confirmed for NDV antigen expression.

93-100% NDV efficacy (D28/D42) was demonstrated against a velogenic NDV challenge for both *in ovo* and subcutaneous routes of administration in SPF birds.

80-83% MDV efficacy (D5/D54) was demonstrated against a virulent MDV challenge for both *in ovo* and subcutaneous routes of administration in SPF birds

100% NDV efficacy (D33/D47) was demonstrated against a velogenic NDV challenge for the *in ovo* route of administration in broilers.

Genetic & phenotypic stability of this recombinant vaccine was demonstrated for both *in vitro* and in *vivo* passages.

ACKNOWLEDGEMENTS

The authors wish to thank Zoetis ARS (Animal Research Service) for excellent animal work; and the HVT-ND project team and colleagues for help and discussions.

Figure 1. Target antigen NDV protein expression was demonstrated by Immunofluorescence using either NDV anti-serum, or monoclonal antibody against HVT.

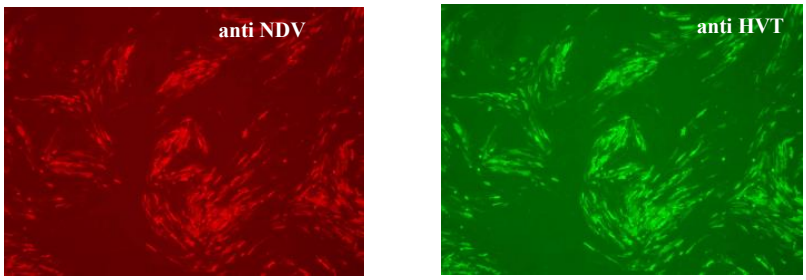

Table 1.

Trt	Vaccine	Birds	Route	NDV Challenge (D28)	% NDV Protected (D42)
T01	Non-vaccinated	SPF	-	No	**NA (40/40)**
T02	CEF cells	SPF	*In ovo*	Yes	**0 (0/40)**
T03	HVT-ND	SPF	*In ovo*	Yes	**93 (37/40)**

Table 2.

Trt	Vaccine	Birds	Route	NDV Challenge (D28)	% NDV Protected (D42)
T01	Non-vaccinated	SPF	-	No	**NA (40/40)**
T02	CEF cells	SPF	Subcutaneous	Yes	**0 (0/40)**
T04	HVT-ND	SPF	Subcutaneous	Yes	**100 (40/40)**

Table 3.

Trt	Vaccine	Birds	Route	MDV Challenge (D5)	% MDV Protected (D54)
T01	Non-vaccinated	SPF	-	No	**NA (30/30)**
T02	CEF cells	SPF	*In ovo*	Yes	**10 (3/30)**
T03	HVT-ND	SPF	*In ovo*	Yes	**83 (25/30)**

Table 4.

Trt	Vaccine	Birds	Route	MDV Challenge (D5)	% MDV Protected (D54)
T01	Non-vaccinated	SPF	-	No	**NA (30/30)**
T02	CEF cells	SPF	Subcutaneous	Yes	**0 (0/30)**
T04	HVT-ND	SPF	Subcutaneous	Yes	**80 (24/30)**

Table 5.

Trt	Vaccine	Birds	Route	NDV Challenge (D33)	% NDV Protected (D47)
T01	Non-vaccinated	Broilers	-	No	**NA (0/12))**
T02	Challenge control	Broilers	-	Yes	**8 (1/12)**
T05	HVT-ND	Broilers	*In ovo*	Yes	**100 (12/12)**

INFLUENCING THE AVIAN MICROBIOTA: USING MRF TO IMPROVE CONTROL OF FOODBORNE ILLNESS ASSOCIATED PATHOGENS

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SUMMARY

Foodborne illnesses continue to be a hot topic of discussion in the poultry industry. The most common bacteria associated with foodborne illnesses are in the genus *Campylobacter*, *Salmonella*, *Clostridium*, and *Staphylococcus*. In 2015, the Foodnet Surveillance report by the Center for Disease Control (CDC) listed that the most common hospitalizations were due to *Salmonella* and *Campylobacter*. From this report, poultry was indicated to be the major source of *Campylobacter* and that the incidence of human infection appears to be on the rise. The same report also indicated that the second most common infection was *Salmonella*, with the most common serotypes being Enteriditis, Newport, and Typhimurium. Enteriditis infections are of interest to the poultry sector due to that sources of infection have been linked to both chicken and eggs. Recently, other *Salmonella* infections have become of interest, such as *Salmonella* Reading, because of an increase in human infection cases. Despite of regulatory programs implemented poultry production systems to try to control contamination issues, foodborne pathogen infections do not appear to be decreasing. Mitigation strategies should include control of pathogenic enteric pathogens pre-harvest, at the processing plant level and at the consumer end. In this article, we discuss the microbiome and the benefits of MRF prebiotic as a possible complement to the pre-harvest food safety program.

THE GUT MICROBIOME

The avian gut microbiome is composed of a balance between bacteria, protozoa, fungi and viruses (1-3). The microbe interactions in the gut are complex and not always understood. Bacteria are overrepresented members in this vast community of gastrointestinal microorganisms and their interactions play an important role in the host (4). The complexity of the avian microbiota allows for nutrient utilization, homeostasis, growth, gut immunity and overall well-being. Culturable techniques were a limiting factor of understanding all the bacterial populations and their effect on each other (5). Recently, molecular techniques and genome sequencing have added more information about microbial communities and their dynamics (3, 4, 6). Bacterial ecosystems continue to be explored and detailed as newer methods of diagnostics are developed, validated and high throughput gene sequencing becomes more affordable. However, there is still limited understanding of the interactions between these gut organisms and the host; as well as the capabilities of the organisms and their function.

Commercial poultry species are diverse with different production systems, environments, stresses and diets. The microbiota of poultry tends be a research focus due to their agricultural and food safety significance. Previous research has demonstrated that bacterial communities are different even within same species; for example, the microbiota of wild turkeys is different from that of a commercial turkey (7). Furthermore, the microbial communities between poultry species, particularly of chickens and turkeys, shows only a 16-19% similarity (8). Even within a single bird there are unique profiles of bacteria present along the different parts of the gastrointestinal tract (6, 8). The ceca contain the highest density of microbes within the gastrointestinal tract and these can be as high as 1011 CFU/g of cecal content. The density and diversity of microbes, specifically bacteria, in the ceca supports beneficial microbial fermentation which plays an essential role for water and salt absorption, non-digestible carbohydrate fermentation and uric acid degradation, as well as the microbial production of volatile fatty acids (9). Most of the cecal microbiota is dominated by *Firmicutes*, *Bacterioidetes* and *Proteobacteria*, which previous studies have shown to represent > 90% of the bacterial phylum (2, 3, 8). However, *Firmicutes* are the predominant phylum; particularly in the ceca of the chicken and *Bacteroidetes* in the cecum of the turkey (8). Bacterial groups in the intestinal tract of poultry, whether commensal or pathogenic, tend to be influenced by a variety of factors. Changes in the microbiota of poultry have been correlated with poor intestinal health, susceptibility to other diseases and gastrointestinal colonization opportunistic and pathogenic bacteria.

This shift in microbiota diversity is of major concern due to possible contamination with bacteria such as *Salmonella* spp. and *Campylobacter* spp. in poultry products used for human consumption (10, 11).

INFLUENCING BACTERIAL POPULATION IN EARLY GASTROINTESTINAL TRACT DEVELOPMENT

Microbiota and their metabolites can influence health and disease of the host directly and indirectly. This can be achieved through physiological changes in the barrier functionality as well as changes in modulating the innate and adaptive immune system responses (12). The disruption of a healthy microbiota is often associated with a shift in the core profile and abundance microbial species, otherwise known as dysbiosis. Some factors that contribute to dysbiosis include disease, antibiotics therapy, environmental factors, toxins, feed access and variability of feed ingredients (12-14).

Newly hatched chicks have a relatively sterile gastrointestinal tract with some early colonizers deriving from parent to progeny, but the vast diversity in the microflora is influenced by factors post-hatch (15). Contact from the bird's immediate surroundings and placement in the farm, allows the chick to develop the intestinal flora (16). This development is mainly due to the differences in bacteria ingestion from hatching debris, surrounding environment, handling, the hatchery facility as well as feed, litter and water at the farm (17-19). Establishment of a mature intestinal microbiota in early development has shown to prevent pathogen infections in poultry (20).

Early gastrointestinal maturation and seeding the gut with beneficial bacteria has been shown to significantly reduce the rate of infection of pathogenic bacteria such as *Salmonella* and *Campylobacter* (21, 22). Beneficial microbes can aid in the gastrointestinal lining barrier protection. Conditioning and rehabilitation can occur through products that have shown to be supportive of the gastrointestinal environment and the microflora. Some of these 'functional' products include prebiotics, probiotics, mannan-rich fractions (MRF), enzymes, organic acids, vaccine programs, essential oils and spices among others (23).

MANNAN-RICH FRACTION'S ROLE IN GUT HEALTH

Among an array of beneficial feed additives, prebiotics have gained popularity due to their well-known roles in improving gastrointestinal health and performance parameters. There are different prebiotics well known for their role in in growth and performance improvement. From the list of current prebiotics, mannan oligosaccharides (MOS) are particularly interesting in the food animal industry due to their capability of prevention of colonization of pathogenic bacteria as well as improving feed conversion, average weight gains and livability (24, 25). Research has also demonstrated the ability to prevent pathogenic bacterial colonization through competitive exclusion and by promoting the increase of lactic acid producing bacteria; as well as changes in production of volatile fatty acids (26). Hence, making the gastrointestinal environment more suitable to beneficial bacteria; particularly, Lactobacilli spp. in the mid and upper intestinal tract. Further research in the area of MOS nutrigenomics led to better identification of the active minding site of MOS from the outer layer of a specific strain of yeast – S*accharomyces cerevisiae*. These second-generation MOS products are known as mannan-rich fractions (MRF). MRF's capability of providing effective immune response while improving nutrient utilization has been described in poultry species repeatedly (24, 25, 27). MRF prebiotics have act differently than other prebiotics in that they possess high affinity for type-1 fimbria bacterial ligands (22, 28). This attachment allows for adsorption of the bacteria to the MRF molecule instead of the intestinal epithelium and therefore aids in decreasing bacteria with type-1 fimbria like *Salmonella* and *E. coli* from colonizing intestinal cells. Bacterial binding to MRF are also known for immunomodulation through increasing local immunoglobulin A production, increasing mucus production and helping decrease tissue inflammation (29, 30). Furthermore, dietary supplementation of MRF research has shown to help alter the bacterial communities and reduce unfavorable bacteria such as *Salmonella*, *E. coli*, *Clostridium* spp. and *Campylobacter* (13, 21, 28, 31, 32).

MRF'S POTENTIAL IN COMPLEMENTING FOOD SAFETY PROGRAMS

Food safety programs should comprise of a combination of risk mitigating strategies at pre-harvest, post-harvest and consumer education. Pre-harvest control of pathogenic bacteria in live animals has shown to reduce *Salmonella* and *Campylobacter* at the farm level (33, 34). Dietary interventions that modulate the microbiota of the bird from an early start would help decrease the colonization of bacterial pathogens later down the harvest chain. Acceleration of the chick's microbiota and intestinal capabilities play an essential role in decreasing the risk of undesirable bacterial populations establishing in the gastrointestinal tract. Seeding the intestinal tract with microbial communities post-hatch can be influenced

by parent stock microbiome establishment or *in ovo* interventions or handling (15, 35). Maintenance of the beneficial microorganisms can continue to be modulated in field settings throughout the life of the flock through proper environment, management, water acidification and cleaning; as well as feed ingredient strategies well known for benefiting intestinal health. MRF supplementation has sown to reduce the number of harmful bacteria in the intestinal tract particularly post-infection (13, 22).

CONCLUSION

In the age of highlighting the animal food industry from a negative aspect, foodborne associated bacterial infection in people have further impacted the poultry industry. Finding alternative approaches, particularly those associated with more natural alternatives, in combination with good management programs and continued education of the consumer will be key in reducing foodborne bacterial illness. MRF's have been shown to decrease the effects associated with pathogenic bacterial colonization as well as the ability to alter the bird's immunity and physiochemical composition of the gastrointestinal tract (26, 36). As the meat and egg industry moves forward with new guidelines for food safety, it will be of outmost importance to adapt key solutions to prevent and decrease bacterial contamination in poultry products. Feed supplements that focus on improving intestinal health, decreasing disease susceptibility in animals, and stabilization of the microbiome pose a viable alternative as a key part of a food safety program.

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USING THE PICHINDE VIRUS VECTOR TO DELIVER VACCINATION AGAINST TURKEY ARTHRITIS REOVIRUS - *IN VITRO* STUDY

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SUMMARY

In previous studies, Pichinde virus vector (PICV) was developed by reverse genetics technology and was successfully used to provide protection against lethal influenza virus in mice. Later, the PICV vaccine's safety and efficacy were established in a chicken host. In this study, we have developed recombinant PICV vaccines that carry S1 and/or S3 genes of turkey arthritis reovirus (TARV). Currently, there is no commercially available TARV vaccines. Also, the use of live attenuated vaccines may help in the emergence of new TARV mutants. The PICV vector expressing TARV antigenic proteins can be a safe and effective alternative. The developed nine recombinant PICV vaccines carried the wild type S1 and/or S3 from three TARV isolates (SKM73, SKM95, and SKM121) and three vaccines carried codon-optimized S1 and/or S3 of SKM121. All recombinant vector viruses were rescued successfully on BSRT7-5 cells and grew on BHK-21 cells. The inserted segments of TARV were detected using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Direct fluorescent antibody (FA) technique using FITC-conjugated anti-avian reovirus antibodies enabled the detection of the expressed TARV proteins in BHK-21 cells inoculated by recombinant PICV vaccine. The fluorescence was remarkably observed in PICV vaccines that contain genes of SKM121. This work proved the success of the PICV vaccine to express the antigenic proteins from TARV genes. Future in vivo studies will be conducted in turkeys to establish safety and efficacy.

INTRODUCTION

TARVs re-emerged in 2011 (4, 7). The infected turkeys displayed clinical disease in the form of lameness, tenosynovitis, and arthritis resulting in huge economic losses partially due to culling. No commercial vaccines are available to protect turkey flocks against the evolving TARV strains. Some turkey producers rely on the use of autogenous vaccines. However, the evolving of new mutant strains creates a continuous need to update the vaccines regularly. Additionally, there is no live vaccine that can act as a live prime before the injectable killed vaccines.

Since Reovirus is a segmented RNA virus, it is not recommended to use live vaccines as this may help in the emergence of new viral mutants. Using a live and safe vector to deliver a TARV subunit vaccine is a good alternative for using live reovirus vaccines. Recently, a research team developed a PICV vector that can be used to deliver model antigens safely and effectively (1). This arenavirus was first isolated from its natural host Oryzomys albigularis (rice rats) in the Pichinde valley of Colombia, South America (9). Arenaviruses are known to target dendritic cells and macrophages at early stages of infection, making it a potential vaccine vector (2, 3, 5, 6). The engineered PICV vector was recovered by transfection of BSRT7-5 with 3 plasmids: pUC18-P18Lag, P18S-GPC/PR8HA or P18S-GPC/PR8NP, and P18S-GFP/NP. The reverse genetics technique produced the live recombinant PICV (strain 18) with a tri-segmented RNA genome (rP18tri). The rP18tri can carry and express up to two foreign genes and the green fluorescent protein (GFP) can be one of them.

The rP18tri is attenuated both *in vitro* and *in vivo*. It has the ability to induce cell-mediated and humoral immune response (1). Thus, mice immunized with recombinant PICV-hemagglutinin (PICV-HA) developed an immune response that was completely protective against lethal avian influenza virus infection. Additionally, the immune response (both humoral and cell-mediated) increased significantly after booster dose (1). The same group established the efficacy and safety of PICV-HA in a live chicken host (unpublished).

The present study was undertaken to develop monovalent and bivalent recombinant PICV vaccines having TARV genetic segments; S1, S3 or both. This in vitro study is a proof of concept study for the development and recovery of recombinant PICV-TARV vaccines and the expression of the antigenic protein/s.

MATERIALS AND METHODS

Cells and viruses. QT-35 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 50ug/mL penicillin-streptomycin. Baby hamster kidney (BHK-21) cells, BSRT7-5 cells (BHK-21 cells stably expressing T7 RNA polymerase), and LMH cells were grown in minimal essential medium (MEM) (Sigma-Aldrich) that containing 10% FBS, 1 ug/mL Gentamicin, and 50 ug/mL penicillin-streptomycin. Media for BSRT7-5 cells was also supplemented with 1µg/mL geneticin (Invitrogen-Life Technologies). Three isolates of turkey arthritis reovirus (SKM73, SKM95 and SKM121) were isolated from tendon tissue in QT-35 cells.

Pichinde virus plasmids. Three plasmids were used:

1. pP18S1-GPC/MCS (the S1 plasmid) encodes the glycoprotein GPC and a multiple-cloning-site (MCS) to clone the gene of interest.
2. pP18S2-MCS /NP (the S2 plasmid) encodes the nucleoprotein NP and a multiple-cloning site (MCS) to clone the gene of interest.
3. pP18L plasmid expresses the full-length antigenomic strand of the rP18L segment under the control of T7 promoter and does not contain a specific site to clone any foreign genes (1).

Plasmids were obtained from Dr. Ly's laboratory at the University of Minnesota, Saint Paul, MN, USA.

Preparation of vectors and gene inserts. Genomic RNA was isolated and full-length open reading frame (ORF) of S1 and S3 segments of three different TARV isolates (SKM73, SKM95, and SKM121) were amplified. Additionally, the S1 and S3 ORF sequence of SKM121 were submitted to another laboratory to obtain their codon-optimized versions. The PCR amplified product (S1 and S3 genes of all three isolates) and the plasmids of the PICV (pP18S1-GPC/MCS and pP18S2-MCS /NP) were restriction digested.

Cloning and transfection. The S1 and S3 gene inserts were ligated in MCS region of the plasmid pP18S2-MCS/NP and pP18S1-GPC/MCS, respectively by using T4 DNA Ligase (5 U/µL) (Thermo Fisher Scientific Catalog#EL0011, Waltham, MA, USA). The ligation reaction mix were used to transform competent bacterial cells (DH5α) followed by selection using ampicillin antibiotic. The plasmids were used to transfect BSRT7-5 cells in the combination shown in Table 2 using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific Catalog#L3000008, Waltham, MA, USA) following manufacturer's instruction with minor modifications. After 48, 72 and 96 h post transfection, cell supernatants were collected and were stored at -80°C. In this way, the recombinant viruses were rescued. The resultant viral recovery was confirmed by observing the green fluorescence of GFP in the inoculated cell culture. The yielded virus was then grown in BHK-21 cells and the GFP green fluorescence was observed. Presence of reovirus genes was confirmed by gene specific PCR.

Detection of reovirus antigen expression. The expression of σC and σB protein in BHK-21 cells infected with recombinant trisegmented PICV vaccine vector was verified by direct fluorescence antibody (FA) assay using polyclonal FITC-conjugated anti-avian reovirus antibodies (National Veterinary Services Laboratory, Reagent#680-ADV, Ames, IA, USA).

RESULTS

Plasmid transfection and virus rescue. The viable recombinant PICV vector viruses were successfully rescued following transfection of BSRT7-5 cells with three plasmids (L, S1 & S2) in the combinations shown in. The GFP expression was observed 48-72 h post transfection in cells transfected with at least one GFP containing plasmid (All monovalent vaccines in Table 2). The GFP-expressing foci grew in their size over the time course of transfection. The supernatants were collected from transfected BSRT7-5 cells and were used to infect BHK21 cells. Strong GFP expression was detected in supernatant infected BHK21 cells at 24-48 hpi, as observed under fluorescence microscopy. This indicate the rescue of viable viruses. At every rescue attempt, we obtained infectious viruses at 48-72 h post transfection. The vaccines showed minor GFP fluorescence in QT-35 and LMH cells at 96 hours after inoculation. The bivalent vaccines having two TARV genes on both plasmids did not produce any green fluorescence.

Recombinant PICV vector viruses expresses reovirus antigens. The strong GFP expression by infected BHK21 cells indicated successful rescue of recombinant viruses. To determine whether reovirus antigens (σC and σB) were expressed by the recombinant viable PICV viruses, we infected the BHK21 cells with transfection supernatant and then at 48 hpi conducted a direct Fluorescence assay (IFA) using polyclonal FITC-conjugated anti-avian reovirus antibodies. The grown PICV vaccines on BHK-21 showed varying degrees of fluorescence in the FA assay. The monovalent and bivalent PICV vaccines that contained either S1, S3 or both showed fluorescence in FA test on BHK-21 cells. Although we have not conducted any procedures for quantifying that amount of fluorescence, PICV vaccines that contained SKM121 gene segments showed a

remarkably higher degree of fluorescence, particularly the bivalent PICV vaccine that contained codon-optimized S1 and S3 segments.

DISCUSSION

Using PICV as a vector to deliver TARV vaccine can be a good alternative to the use of live attenuated vaccines, which may lead to the emergence of mutant strains. PICV recombined with the hemagglutinin gene of influenza showed the ability to initiate effective humoral and cell-mediated immune responses and gave protection against lethal influenza in mice (1) and was shown to be safe and effective in chickens (Ly, personal communication). The purpose of this in vitro study was to develop the PICV-TARV recombinant vaccine that can carry S1 and/or S3 genes of TARV. We also aimed at successfully recovering the PICV after the insertion of the TARV genomic segments in the PICV plasmids, detection of the inserted genes and expression of the recombinant antigenic proteins.

In this work, we developed 12 different recombinant PICV vaccines. We used the wild type genes from three different TARV isolates in addition to codon-optimized genes from one TARV isolate. This design should help in finding an optimum TARV candidate to be used in developing a vaccine. These TARV isolates were selected on the basis of their high pathogenicity as described in the clinical history of the disease in turkeys. The use of codon-optimized genes was recommended by the research team, who initially developed the PICV as a vaccine vector.

The recovered PICV recombinants grew well in BHK-21 cells but showed minimal growth on QT-35 and LMH cells as shown by GFP green fluorescence. The PICV vaccines that contained one TARV gene had GFP and viral growth could be easily determined. The green fluorescence test could not be used to determine the growth of the double recombinant (PICV that contained both S1 and S3 genes of TARV because they did not express GFP). We assume that these viruses grew the same way the other monovalent vaccines grew because their plasmids and transfection were done under the same condition. The detection of the inserted genes and protein expression were helpful in detecting the successful bivalent PICV recovery and growth. Using RT-PCR to detect the inserted TARV genome in the recovered viruses helped the detection of the success of recovering the recombinant PICV that contained either S1, S3, or both.

We wanted to determine the protein expression by the inserted gene in the PICV vaccine. We used the direct fluorescent antibody technique (FA) using FITC-conjugated anti-avian reovirus antibodies for this purpose. The FA procedure included dehydration and long fixation with acetone. These two steps guaranteed the elimination of GFP fluorescence and the permeabilization of cell membrane. We wanted to eliminate the GFP fluorescence to be sure that any green fluorescence after FA will be due to FITC. The permeabilization of the cell membrane enabled the entrance of the FITC-conjugated antibody to attach to the intracellular recombinant viral proteins. Although we did not have a quantitative method to measure the amount of the expressed protein, we could subjectively observe that the fluorescence produced by vaccines recombined with SKM121 genes was more than those of SKM73 and SKM95. The bivalent PICV that contained codon-optimized S1 and S3 of SKM121 showed the best fluorescence as compared with all other vaccines. The observation of the fluorescence, after FA in BHK-21 cells inoculated with the bivalent PICV vaccines, was indicative of the growth of these viruses which did not have any GFP fluorescence after transfection.

In conclusion, the PICV bivalent vaccines carrying codon-optimized genes of SKM121 isolate showed the best results. PICV recombined with codon optimized genes of SKM121 will be used for the future in vivo study in turkeys to study the efficacy and safety of the developed PICV-TARV recombinant vaccines.

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OUTBREAKS OF ASPERGILLOSIS IN YOUNG TURKEYS PROBABLY DUE TO HATCHERY CONTAMINATION

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SUMMARY

Aspergillosis is a mycotic disease of birds in general and poultry in particular such as turkeys, chickens, and waterfowl. The disease in young poultry is commonly called "brooder pneumonia." The disease is caused by members of the genus *Aspergillus*, most commonly by *A. fumigatus* and *A. flavus*. The disease is manifested commonly by respiratory signs due to presence of lesions in the air sacs, lungs, and syrinx and occasionally by neurological signs due to lesions in the brain.

A retrospective study performed by searching the CAHFS electronic data-base on the cases diagnosed with aspergillosis in turkeys submitted to the Turlock and Tulare laboratories for the years 2018 and 2019 identified 96 cases. An assumption was made that the turkey poults submitted between the ages day-old and seven days and diagnosed with aspergillosis were probably due to the hatchery contamination. Forty out of 96 such cases were identified. These poults had a few small pale yellow foci ranging in size from 1-2 mm to 3-4 mm in the air sacs and lungs. Older poults between 8 and 28 days (31/96 cases) had a few to numerous larger pale yellow nodules throughout the lungs and air sacs and occasionally in the brain. Many of these cases were most likely were originally diagnosed in poults when they were between one-day and seven-days old. Microscopically the lesions were composed of giant cell granulomas associated with mycelia of *Aspergillus* spp. *A. fumigatus* was most commonly isolated from the lesions.

A RETROSPECTIVE STUDY OF BORDETELLOSIS (TURKEY CORYZA) IN CALIFORNIA TURKEYS: 2009-2019

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SUMMARY

Bordetellosis is a highly contagious upper respiratory infectious disease of poultry, especially turkeys caused by the bacterium *Bordetella avium*. The disease is characterized by respiratory signs such as open-mouth breathing, lacrimation, swollen infraorbital sinuses, increased mucus in the trachea, and in severe cases collapsed trachea. The disease most commonly occurs in two to six-week-old turkeys. Morbidity in a flock can be as high as 80 % to 100 % but mortality is usually less than 10 %. Microscopic lesions in the upper respiratory tract consist of presence of bacteria attached to the cilia in acute cases, deciliation, dilated mucous glands, lymphoplasmacytic inflammation, and in chronic cases squamous metaplasia of the mucosal epithelium. *B. avium* can be isolated easily especially in acute cases from the trachea and serology is useful for diagnosis.

A retrospective study performed by searching the CAHFS electronic database on the cases diagnosed with bordetellosis in turkeys submitted to the Turlock and Tulare laboratories between 2009 and 2019 identified 397 cases. The number of cases per year ranged from 7 to 62 with an average of 36 cases per year. The criteria for selection of the cases of bordetellosis was isolation of *B. avium* most commonly from the trachea and/or sinus, air sacs, and lungs. Serology in birds 10 weeks of age or less, as well as, compatible microscopic lesions, were also used as criteria for diagnosis.

Most common clinical history in birds with bordetellosis was respiratory signs, swollen sinuses, lacrimation, and increased mortality. The age of birds ranged from two weeks to 10 weeks with an average age of 5.9 weeks. Gross lesions in the trachea ranged from increased mucus in the lumen to collapse of the cartilaginous rings and increased mucus in the sinuses and conjunctiva. Microscopic lesions included bacteria attached to the cilia in acute cases and deciliation, lymphoplasmacytic inflammation of the mucosa, attenuation of the glands, and squamous metaplasia of the epithelium in chronic cases. Sinus/turbinates and conjunctiva were also affected with lymphoplasmacytic inflammation of the mucosa with occasional fibrinosuppurative inflammation extending in to the adjacent tissues in the nasal passages. Concurrent diseases such as colibacillosis, poult enteritis, ORT, aspergillosis, crop mycosis, HEV, etc., were also diagnosed in many cases.

INFECTIOUS CORYZA – CLINICAL IMPACT, METHODS OF CHARACTERIZATION, AND VACCINATION

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OVERVIEW

Infectious coryza, a respiratory disease that occurs where-ever chickens are raised, is caused by *Avibacterium paragallinarum*. The most prominent features of infectious coryza are an acute inflammation of the upper respiratory tract including involvement of nasal passage and sinuses with a serous to mucoid nasal discharge, facial edema, and conjunctivitis (Figure 1). The disease is characterized by poor growth performance and a marked reduction (10–40%) in egg production in layers (Blackall & Soriano-Vargas, 2020).

ETIOLOGY

Infectious coryza is caused by *A. paragallinarum*, a Gram-negative bacterium belonging to Pasteurellaceae family (1). Culture on blood agar plates, require cross-streaked *Staphylococcus epidermidis* culture as a feeder of nicotinamide adenine dinucleotide (NAD; Figure 2). NAD independent isolates have been reported from South Africa, Mexico, Peru and Korea (2).

METHODS OF CHARACTERIZATION

The hemagglutinating antigens (Figure 3), are the main structure for the serologic classification of *A. paragallinarum* isolates. Page scheme recognizes serovars A, B, and C. Kume scheme consists of serogroups A, B, and C which match the three Page serovars. The nine currently recognized Kume serovars are A-1, A-2, A-3, A-4; B-1; C-1, C-2, C-3 and C-4. The distribution of Kume serovars differs from country to country (Figure 4).

Page and Kume serotyping of 11 Dutch isolates showed the presence of serovars A-1, A-2 and B-1 (8).

While a multiplex PCR assay and a PCR-RFLP assay to recognize the Page serovars was proposed (17), further studies have indicated significant problems in this approach (16, 23) and the assay cannot be recommended as a replacement for Page serotyping.

Particularly, serovar C-1 was identified in 1975 in Fukushima, Japan. In 2000, this serovar was was identified in Cotopaxi and Pichincha municipalities, Ecuador (3). Since 2008, serovar C-1 has been isolated from layer hens at Jalisco and Puebla states, Mexico. All isolates included in that study, were from infectious coryza-vaccinated flocks (14). An identical ERIC-PCR genotype, a repeatable and robust technique for genotyping of this bacterium (21), was obtained among Ecuadorian and Mexican serovar C-1 isolates, suggesting a clonal relationship (9). Furthermore, in a phylogenetic analysis of 16S rRNA and hagA gene, all Ecuadorian and Mexican isolates were clustered into a separate genetic lineage. All the isolates included in that study were most distantly related to reference strain serovar C-1, H-18, of *A. paragallinarum* (Figure 5).

Recent outbreaks of infectious coryza have been reported in California, USA (6, 7).

VACCINATION

The protective antigens of *A. paragallinarum* have not been definitively identified. However, the hemagglutinin antigens have been proposed as the main pathogenic and immunogenic structures (24). Thus, hemagglutination-inhibition antibody levels of both vaccinated and passive-immunized chickens have been closely correlated with protection against clinical signs and nasal clearance of the challenge organism. Furthermore, the cross-protection and hemaglutination-inhibition antibody titers of inactivated infectious coryza vaccines are dependent on the serovars included in the vaccines (19). Infectious coryza is relatively unique among common bacterial infections in that a bacterin (inactivated whole cell vaccine) is protective against the disease when the bacterin is adequately prepared (2).

In Mexico, differences in the protection conferred by four commercially available infectious coryza vaccines against an isolate serovar C-1 were observed. Only one of the four evaluated, trivalent infectious coryza vaccines gave a good level of protection against challenge with that strain (15).

PATHOGENICITY

The pathogenicity of *A. paragallinarum* can vary according to factors such as the growth conditions, passage history of the isolate and the state of the host. There is now considerable specific evidence of variation in pathogenicity amongst *A. paragallinarum* isolates (2). The Kume serovars A-1, A-4, C-1, C-2, and C-3 showed higher virulence than other serovars (18, 22). Similarly, variation in virulence among serovar B-1 strains from Germany, Panama, Ecuador, and Mexico, was recorded (13).

In Panama, severe infectious coryza outbreaks in broiler breeders with isolation of *A. paragallinarum* serovar B-1, were characterized by an up to 45% drop in egg production, increased mortality (from 0.2% per week up to 1.6% per week), and a significant reduction in activity in males (4).

In Mexico, the pathogenicity, virulence, and histologic outcome of SPF experimental chicken infection with a well-characterized isolate ESV-135 was investigated (22). Slight to severe, extensive hemorrhages were observed in the lumen, mucous membranes, and lamina propria of the nasal cavity and infraorbital sinus in most of the chickens inoculated (Figure 6).

Coinfection of *A. paragallinarum* and other bacterial agents may occur, for example *Ornithobacterium rhinotracheale*, and these outbreaks could be more severe than single infections (12). Similarly, a synergistic effect between *A. paragallinarum* and infectious bronchitis virus, resulted in exacerbation of clinical signs and increased mortality in broiler chickens with neurologic signs in California (7).

ANTIMICROBIAL SENSITIVITY

The antimicrobial sensitivity of 66 *A. paragallinarum* isolates from Ecuador, Mexico, Panama, and Peru was determined (11a). All isolates were sensitive to amoxicillin-clavulanic acid, ampicillin, and fosfomycin. Authors suggested that antimicrobial sensitivity differences may exist based on the geographical origin of isolates. Susceptibility patterns of American isolates were similar to those seen for most of the antimicrobials (amoxicillin, ampicillin, erythromycin, lincomycin, neomycin, and streptomycin) in recent studies in Taiwan (11) and Thailand (5). A notable feature is the markedly lower levels of sensitivity in Thai isolates to trimethoprim-sulfamethoxazole (33%), compared to that seen in the Ecuadorian (69%) and Mexican (89%) isolates.

In a study that included 44 field isolates from 24 different Dutch commercial farms with infectious coryza, between 2008 and mid-2017, with exception of tetracycline, results showed relatively good susceptibility to antimicrobial agents that are recommended for the treatment of infectious coryza in the Netherlands (10).

CONCLUSION

Bacterins are needed to reduce the economic losses associated with infectious coryza. As these bacterins are serovar-specific, a knowledge of serovars in a region is needed for an effective vaccination program.

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Figure 1. Chicken after nasal exposure to *Avibacterium paragallinarum*. Conjunctivitis, nasal discharge, facial and submandibular edema, is observed.

Figure 2. *Avibacterium paragallinarum* colonies showing satellitic growth.

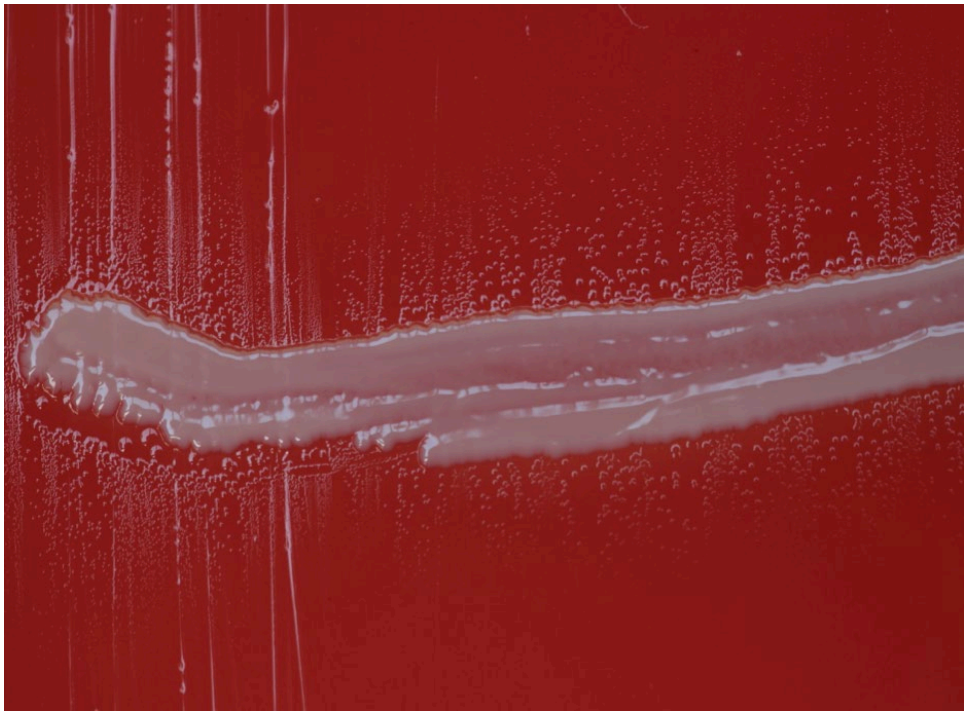


Figure 3. *Avibacterium paragallinarum* adhered to a chicken red blood cell.

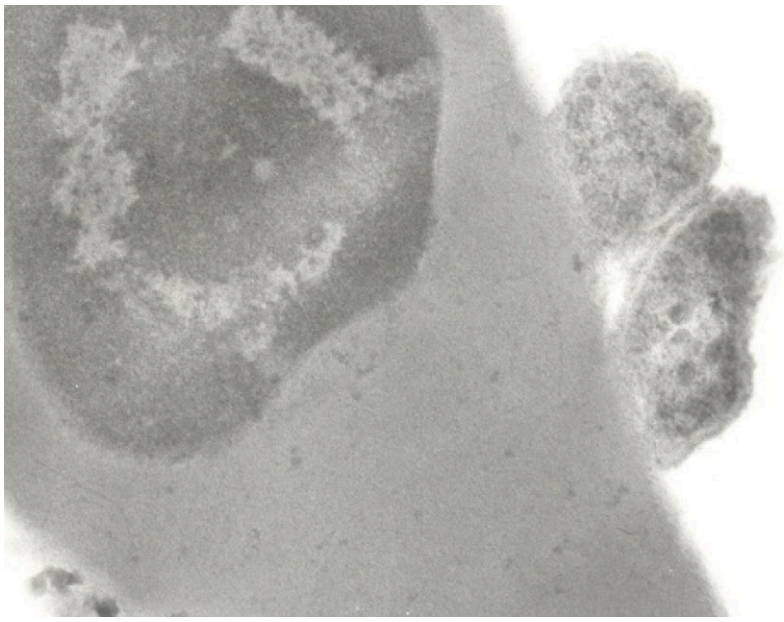


Figure 4. Distribution of Kume serovars of *Avibacterium paragallinarum*.

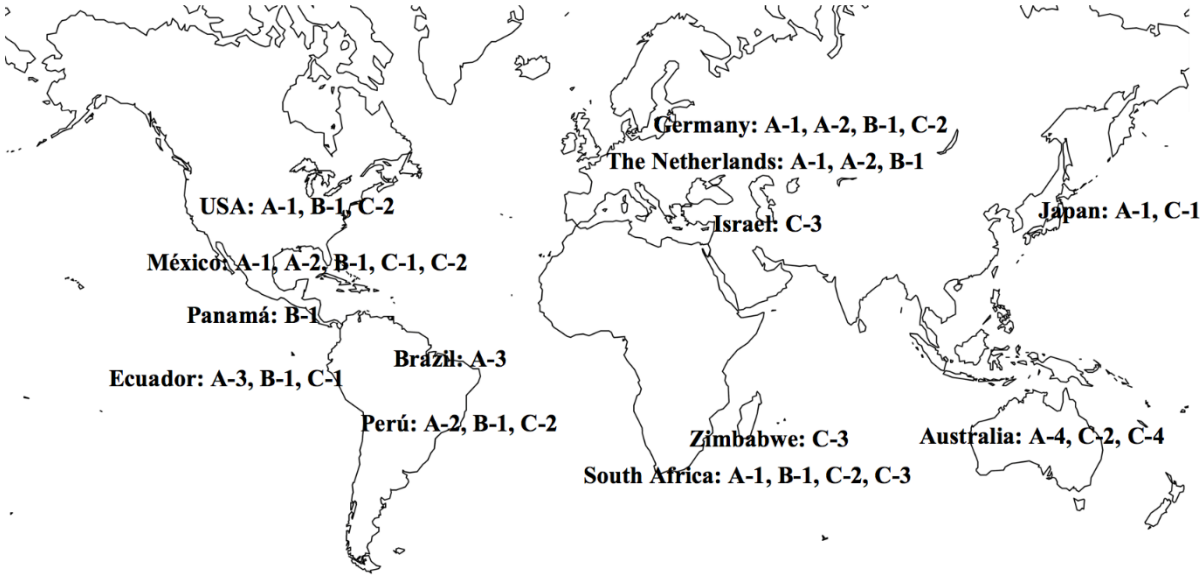


Figure 5. Phylogenetic relationship of *A. paragallinarum* isolates from Ecuador (Lavetec) and Mexico (ESV).

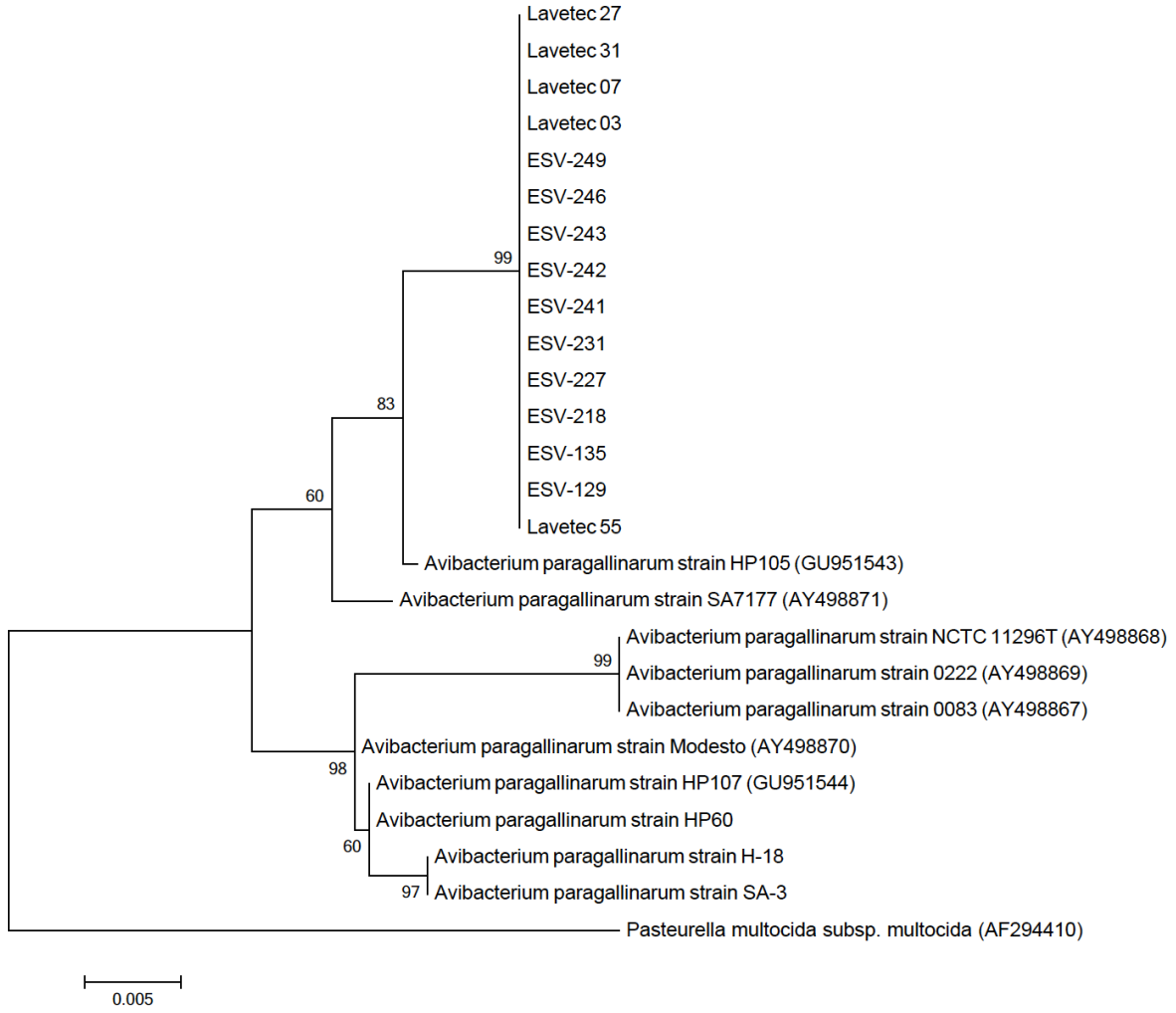
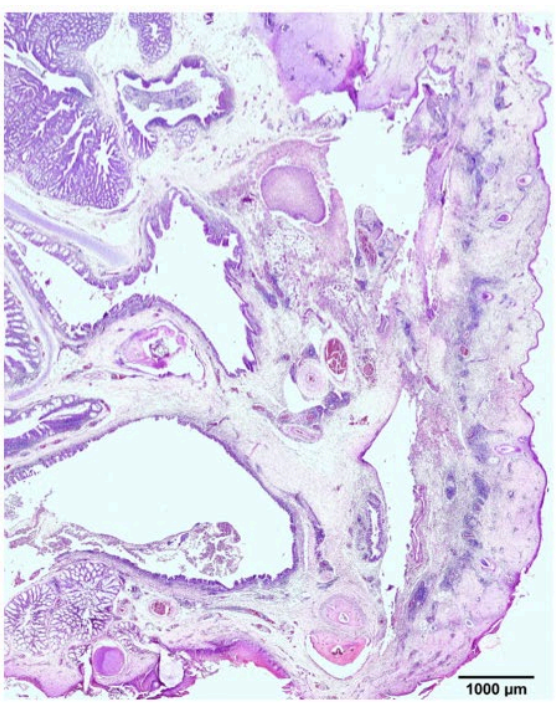
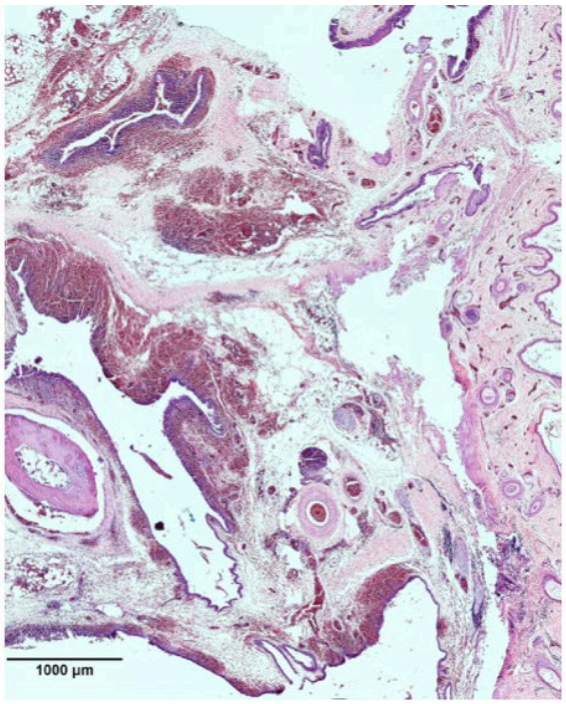


Figure 6. Histologic sections of chickens after nasal exposure to *A. paragallinarum* serovar C-1. Conspicuous hemorrhages in the mucosal membranes of nasal passages after 96 hr post exposure.





DEVELOPMENT OF A FLOW CYTOMETRIC ASSAY TO DETECT PHAGOCYTOSIS AND OXIDATIVE BURST OF HETEROPHILS AND MACROPHAGES OF BROILER CHICKENS

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SUMMARY

The innate immune response is critically important for maintaining the health of chickens. Innate immune cells such as heterophils and macrophages have different antimicrobial mechanisms against pathogens. Synthetic oligodeoxynucleotides (ODN) containing unmethylated cytosine phosphodiester guanine (CpG) motifs stimulate these cells against a variety of viral, bacterial, and protozoan diseases in different animals including poultry. Objectives of this study were; to develop and optimize two-colored flow cytometry based assay to detect phagocytosis and oxidative burst activity of heterophils and macrophages and utilizing this technique to explore immune stimulatory activity of CpG-ODNs. Protocols of Phagoburst® and Phagotest® were modified to examine oxidative burst and phagocytosis activity respectively. Chicks were injected with CpG-ODN or saline. Whole blood collected into heparin tubes were processed according to the modified protocol. It was demonstrated a higher oxidative burst activity in protein kinase-C ligand Phorbol 12-myristate 13-acetate (PMA) stimulated heterophils (60-70 %) than opsonized *E. coli* stimulated heterophils (10-20%). Oxidative burst was higher in heterophils (60-70%) compared to monocyte/macrophages (<10%). Phagocytosis of heterophils (30-60%) and macrophages (30-35%) were higher in CpG-ODN treated group compared to the control group. Blood samples collected after 48 hours of CpG-ODN exposure (five minutes stimulation with PMA, *in vitro*), showed about 60% of oxidative burst activity compared to saline exposure (19%). Here we have optimized the flow cytometry-based assays to measure phagocytosis and oxidative burst activity of heterophils and macrophages of chickens. These assays will be useful tools to study the effects of various immune stimulants and vaccines in chickens.

INTRODUCTION

Phagocytic cells such as heterophils and macrophages perform critical roles in chicken innate immune response against infectious agents. Chicken heterophils are the functional homologous to mammalian neutrophils. These granulocytic phagocytes kill invading pathogens by producing reactive oxygen species (oxidative burst), releasing antimicrobial products (degranulation) following phagocytosis. In oxidative burst, activated NADPH oxidase assists in generating superoxide anion (O2-) then converted into hydrogen peroxide (H2O2) by either spontaneous dismutation or superoxide dismutase (SOD). Both O2- and H2O2 are further transformed into highly reactive and toxic hypochlorous acid (HOCL), hydroxyl radical (OH-) by myeloperoxidases. Since chicken heterophils lack myeloperoxidases, it creates a comparatively weaker oxidative response than the mammalian neutrophils (2, 5, 11). Macrophages are highly phagocytic cells and capable of responding to different chemical stimuli. Further, they are widely distributed and play a crucial role in homeostasis and defense against pathogens. Upon recognition of pathogens by surface receptors and other mechanisms, these cells react within 3 to 6 hours and initiate antimicrobial mechanisms. Studies have been demonstrated that DNA containing CpG and their synthetic equivalent; oligonucleotides containing CpG (CpG-ODN) have immune stimulatory properties in vertebrates as it can act as pathogen associated molecular patterns (PAMPs) (6). CpG-ODN is recognized by toll-like receptor (TLR)-9 in mammals and TLR-21 in poultry. Heterophils, monocytes, and macrophages can directly respond to CpG-ODN in poultry (3). We have recently demonstrated immunostimultory effect of CpG-ODN in chickens using different induction routes (in-ovo, intrapulmonary and intramuscular) (3, 4).

Measuring the functional capacity of the different phagocytic cells will be a useful indicator of the bird's immune responses. Flow cytometry based analysis has been recognized as one of the most

treasured tools as it has the ability to identify the expression of multiple antigens per cell and clearly distinguish cells from each other (9). Considering that, leukocyte common antigens such as CD45 (a membrane glycoprotein) can be used to detect all leukocytes in a sample. Besides, immune cell specific antigens, which facilitate to differentiate them from other cells (1). However, only limited studies have been performed on the functional analysis of avian phagocytes. In the present study, we focused to optimize two-color flow cytometry based assays to quantify oxidative burst and phagocytic activity of heterophils and macrophages in chickens. We exposed chickens to CpG-ODN and measured phagocytosis and oxidative burst activity of heterophils and macrophages in chickens to evaluate their antimicrobial ability *ex vivo*.

MATERIALS AND METHODS

Optimization of two-color flow cytometry based assays. Heparinized whole blood collected from chickens were used in the study. The oxidative burst activity of whole blood was measured using Phagoburst kit (8). Opsonized *Escherichia coli* and protein kinase-C ligand Phorbol 12-myristate 13-acetate (PMA) were used in this commercial kit. Leukocytes were stimulated with *E. coli* or PMA for different periods (two, five, and 10 minutes) prior to adding dihydrorhodamine (DHR) 123 (fluorogenic substrate), which reacts with reactive oxygen species (ROS). Mouse anti-chicken CD45 (APC) fluorescent-labeled antibody and R-Phycoerythrin (PE) labeled anti-chicken monocyte/macrophages specific antibody were added to detect heterophils and macrophages in the total leukocytes. 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), and anti-chicken monocyte/macrophages (PE) were added (7, 10). 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).

Stimulation of leukocytes in chickens by administering CpG-ODN. The CpG-ODN (TCGTCGTTGTCGTTTTGTCGTT2007), which is free of endotoxin and produced with a phosphorothioate backbone (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) into one to three week old chicks. Blood samples were collected after 48 hours of CpG-ODN induction. Heparinized whole blood was subjected to above flow cytometric analysis.

RESULTS

Our results of oxidative burst activity revealed that heterophils stimulated with PMA for two and five minutes have higher oxidative burst activity (60-70 %) compared to cells stimulated with opsonized *E. coli* (10-20 %). Furthermore, heterophils showed higher burst activity (60-70%) compared to monocyte/macrophages (<10%). Phagocytic activity of heterophils (30-60%) and macrophages (30-35%) were measured following stimulation of leukocytes with opsonized *E. coli* (FITC labelled). Oxidative burst of heterophils collected from chickens administered with CpG-ODN (five minutes stimulation with PMA) was higher (60%) compared to control birds that received saline (19%).

DISCUSSION

It was evident that oxidative burst activity of heterophils could be stimulated and measured using this flow cytometric assay, although heterophils lack myeloperoxidases. Highest level of oxidative burst activity of heterophils was detected within five minutes of incubating cells with PMA, as expected since heterophils have the highest killing ability of pathogens. Our results demonstrated that CpG-ODNs are potent immune stimulator and it also increases oxidative burst activity of heterophils. We were able to successfully optimize two-color flow cytometry based assays for functional analysis of phagocytic cells in whole blood of chickens. In addition, this assay can be applied to study immune responses to immune-modulators, vaccines and to measure immunocompetence of different strains of chickens.

CONCLUSION

CpG-ODN is an effective immune stimulant, which can increase oxidative burst and phagocytic activity of heterophils. This two-color flow cytometry based assays can be used for ex-vivo experiments relating to research in immunotherapeutic and vaccine development.

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EXPERIMENTAL STUDIES OF PERSISTENCE AND SPREAD OF CHICKEN ANEMIA VIRUS IN OLDER CHICKENS

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SUMMARY

The horizontal transmission of chicken anemia virus (CAV) has been characterized by the oral-fecal route. There have been no studies of the spread of the virus and associated immune response in older chickens inoculated with CAV by a natural route. Three weeks old SPF chickens were inoculated in two separate groups with the difference CAV isolate. Tissue, cloacal swab, and serum were collected seven-day interval. qPCR was used to detect CAV in DNA extracted from tissues. Swab samples were used to isolate CAV into MDCC-MSB1 cells. Serum samples were used to detect the anti-CAV antibody using a virus neutralization assay. Oral inoculation with CAV resulted in the shedding of the virus via the feces at 7, 14, and 28 days post-inoculation, but no CAV was found at 21 days post-inoculation. Antibody against CAV was demonstrated the first 14 days postinoculation. At 14 days post-inoculation, the virus was consistently found in the highest concentration from the thymus, whereas the bone marrow was low in yield. However, no difference in the viral shedding, viral load, and antibodies response was observed between two CAV isolated groups. These studies indicate that CAV infected-lymphocyte originated from thymus are responsible for the spread of the virus and excreted into feces. Humoral immunity strongly related to inhibiting only in the early phase of CAV infection, while the persistence of CAV showed even after neutralizing antibody was produced.

INTRODUCTION

CAV, the only member of the genus *Gyrovirus* in the family *Anelloviridae*, is a causative agent of chicken infectious anemia disease (CIA). The clinical disease manifests only in young chickens if the infection occurs vertically, but infection remains mostly subclinical in older chickens. It is believed to transmit horizontally to flockmates, presumably via the fecal-oral route. The detection of the viral-tissue distribution in various tissues using quantitative polymerase chain reaction is commonly used. In addition, it may depend on the antibody status of the host. The presence of the virus in the feces in high quantities after parental inoculation was reported. Therefore, the present study aimed to demonstrate the most susceptible tissues, shedding in feces of CAV and anti-CAV antibody, which can be useful to monitor the CAV infection.

MATERIALS AND METHODS

Experimental design. Two isolates of CAV, designated as 1514TW (Group 1) and 1705PT (Group 2) isolates were used. For chicken inoculation, CAV with a titer of 104.5 TCID50/0.1mL was inoculated orally into chickens (0.1mL/chicken). Three-week-old SPF chickens were kept in individual. Thirty-six chickens were divided into three groups and Group 3 was served as an uninoculated control group. Three chickens were sacrificed for tissue dissection and blood samples were collected from the remaining chickens from each group at seven-day intervals.

Viral neutralization assay. The titer of virus-neutralizing (VN) antibodies in serum samples collected from different groups of chickens was measured using the micro-test method according to Imai and Yuasa (2). The antibody titers were determined in two-fold dilutions beginning with a 1:10 dilution.

Quantitative real-time PCR. Tissue samples were used for isolation of whole DNA using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's recommendations. Isolated DNA samples were subjected to real-time PCR analysis using a LightCycler® 480 (Roche, Basel, Switzerland). Duplicate sets of each reaction sample were prepared using a previously described protocol (3). The isolated DNA samples were diluted to 1:100 in order to avoid possible inhibitory effects on efficient PCR reactions by tissue inhibitory substances.

Virus isolation from cloacal swabs. Cloacal swabs were suspended into 1 mL of PBS followed freeze-thawing. The supernatants were collected after low-speed centrifugation and were inoculated in MSB1 cells (5).

RESULTS

Antibody response to CAV and CAV quantity. At 7 dpi, VN antibodies of the chosen chickens in both groups were not detected. At 14 dpi, all chickens in both groups had VN antibodies to CAV, ranging from 1:20 to 1:160 dilution (Table 1). The neutralizing antibody titers for Group 1 started higher but stayed at the same level as Group 2 at 21 dpi. The presence of the highest titer of mean VN antibodies of Group 2 was detected at 21 dpi; however it was present at 28 dpi for Group 2. Two chickens from Group 2 were dropped with VN antibodies at the end of the experiment (28 dpi).

CAV load was quantified in the thymus and bone marrow. At 7 dpi, a significantly higher viral load was observed in the thymus of Group 1 when compared with group 2. (Figure 1) The viral loads in the thymus were the highest when compared with bone marrow at 14 dpi. CAV viral load in the bone marrow was lower than thymus throughout the experimental period. The viral load was low at 7 dpi but increased significantly by day 14 in the thymus of Group 1. However, the viral load did not increase in the bone marrow after day 14.

Two out of three cloacal swabs from both groups were positive at 7 dpi (Table 2) and only one cloacal swab sample was positive at 14 dpi. No viruses were recovered from cloacal swab samples of both groups st 21 dpi. CAV was detectable only in chicken no. 3 at 28 dpi.

DISCUSSION

Among the two organs tested, we found that the thymus had a higher viral load than the bone marrow; therefore, thymus should be the best sample for diagnostic purposes (1). The present study showed that CAV was detected from the thymus in both groups even in the presence of VN antibodies throughout the experiment. It's has been suggested that the thymus and spleen sample remained qPCR positive for CAV DNA despite seroconversion of chickens (4). In this study, CAV was sporadically recovered from cloacal swab samples in Group 2, in which the VN antibodies were low. This result may show that the spread of CAV into feces depends on the immune status. In the previous study, it was assumed that CAV-carrying lymphoid cells originating from the thymus are responsible for re-excretion into feces. The oral route of CAV inoculation was a natural route for older chickens, was used here, it may associate with low immune response and transient virus shedding into the feces; thus, this may play an important source of horizontal CAV transmission in the field.

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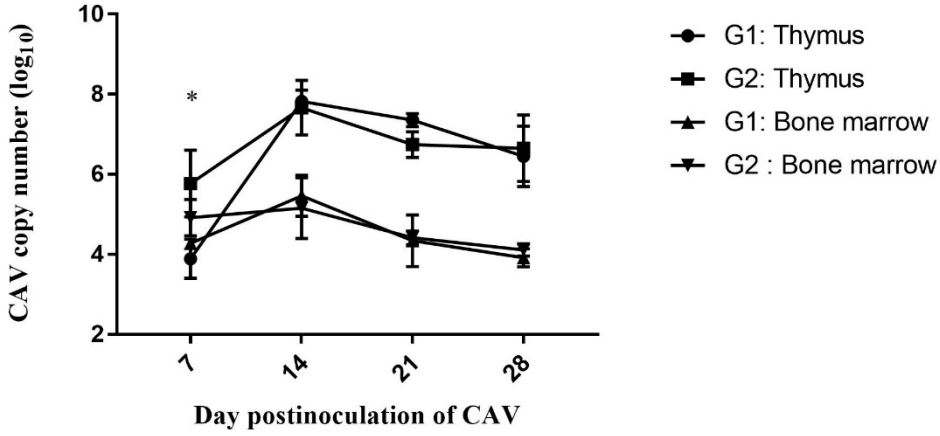
Table 1. Virus neutralizing antibody titers to CAV in chickens inoculated with CAV.

Group	Chicken No.	Neutralizing antibody titers			
		Days after CAV inoculation			
		7	14	21	28
1	1	<10	160	640	1280
	2	<10	160	640	640
	3	<10	40	160	320
	4	<10	160	640	
	)GM(		)88.53(	)452.54(	)640(
2	1	<10	40	640	1280
	2	<10	80	640	320
	3	<10	20	320	160
	4	<10	20	320	
	)GM(		)33.63(	)452.54(	)403.17(

Table 2. Detection of CAV in cloacal swab samples in chickens inoculated with CAV.

Group	Chicken No.	Viral isolation			
		Days after CAV inoculation			
		7	14	21	28
1	1	-	-	-	-
	2	+	-	-	-
	3	+	+	-	-
2	1	+	-	-	-
	2	-	-	-	-
	3	+	+	-	+

Figure 1. CAV viral load in chickens inoculated with CAV.



USUAL AND UNUSUAL MICROSCOPIC LESIONS ASSOCIATED WITH QUAIL BRONCHITIS

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SUMMARY

A northeast Georgia producer submitted two groups of seven-week old Bobwhite Quail (*Colinus virginianus*) with respiratory signs to the Poultry Diagnostic and Research Center Diagnostic Laboratory. Gross lesions included catarrhal or hemorrhagic tracheitis and acute and chronic airsacculitis. Microscopic lesions included fibrinoheterophilic and edematous airsacculitis, necrotizing tracheitis with basophilic intranuclear inclusions, acute hepatic necrosis with histiocytic infiltrates with basophilic intranuclear inclusions and acute multifocal necrotizing bronchitis with suspect intranuclear inclusions.

INTRODUCTION

Quail bronchitis is an acute respiratory disease of young Bobwhite Quail. The disease poses problems for game bird breeders due to high morbidity and mortality and has a worldwide distribution (10). It is caused by quail bronchitis virus/chicken embryo lethal orphan (CELO) virus which are aviadenoviruses. Field reports of morbidity and mortality reach 50% (10) and experimental infections range 0 to 89% depending on age of infection (5).

CASE HISTORY

A northeast Georgia quail producer submitted two groups of seven-week old Bobwhite Quail to the Poultry Diagnostic and Research Center at the College of Veterinary Medicine, University of Georgia with a history of respiratory disease. The first group had seven birds (five alive and two dead) that came from the non-medicated flock. Gross lesions included 5/7 with advanced airsacculitis and 3/7 with catarrhal tracheitis, all had conjunctivitis and the livers had multifocal pale pinpoint areas throughout the parenchyma. The spleens were mottled and enlarged. Three swabs from the air sacs were submitted for bacterial culture. Sections of trachea, liver, air sac, lung, spleen, and heart were collected and fixed in 10% formalin. Group 2 had six live birds from the medicated flock (treated with Neomycin and Tylan prior to submission). Gross lesions included 1/6 with hemorrhagic and mucoid tracheitis and 3/6 with catarrhal airsacculitis. Sections of trachea, liver, spleen and liver were collected and fixed in 10% formalin.

DIAGNOSTIC RESULTS

Group 1 had bacterial culture performed on three swabs from air sacs. *Escherichia coli* was isolated with medium growth and swarmed by *Proteus mirabilis*. Histopathology was performed on both groups. The submitted tissues were routinely processed, embedded, sectioned, stained with Hematoxylin and Eosin, cover slipped and examined by light microscopy. Both groups had tracheas with diffuse ulceration of the respiratory epithelium along with a single trachea that had large basophilic intranuclear inclusions in the respiratory epithelium consistent with adenovirus. Other sections of trachea ranged from multifocal to diffuse lymphoplasmacytic infiltrates with or without heterophils, acute individual cell necrosis of the respiratory epithelium and deciliation of the remaining intact respiratory epithelium. Group 1 also had a section of lung with multiple parabronchi containing cell debris. A secondary bronchus had suspect intranuclear inclusions in the respiratory epithelium that had sloughed into the lumen along with individual necrotic respiratory epithelial cells. The air sac had multiple areas of cell debris, heterophils, and macrophages on the surface of the epithelium. Specials stains of GMS and Brown and Hops failed to identify fungal or bacterial organisms respectively. A liver section had multiple aggregates of macrophages throughout the parenchyma, with a few admixed with fibrin. A few of the foci also had basophilic intranuclear inclusions consistent with adenovirus in dying hepatocytes or cells interpreted to be macrophages. Other lesions shared by both groups included acute multifocal fibrinoid necrosis of the spleens.

Final diagnosis was quail bronchitis with secondary bacterial infection.

DISCUSSION

Quail bronchitis typically manifests itself as an acute severe respiratory disease that can have up to 30-50% mortality and 100% morbidity in quail less than three weeks of age (7, 8). Jack and Reed performed numerous experiments with Bobwhite Quail and quail bronchitis virus (5-8) that demonstrated the virulence of the virus in young birds versus birds infected after three weeks. Olson first described this disease in 1951 (9). There has been only a couple of reports of quail bronchitis in Corturnix Quail group of which Japanese Quail are a member. One report documents the infection in adult quail with a drop in egg production (10-15%), respiratory disease, and eggs lacking pigmentation or soft shelled (2). The other report is in young quail (less than 3 weeks) with inclusions in numerous gastrointestinal sites (11). The microscopic lesions in bobwhites are usually centered on the respiratory system but other organs can be affected. Adenoviral inclusions have been documented in the liver, bursa of Fabricius, ventriculus, and oviduct (1, 3, 4, 10). When dealing with quail in a diagnostic setting, getting the producer to provide numerous affected live birds is key to identifying the inclusions. Submission of tissues like the liver in additional to the respiratory samples will increase the likelihood of a quick diagnosis. Currently there are no commercial vaccines for protection against quail bronchitis so biosecurity is the only means to prevent infection.

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EVALUATION OF AN AVIAN RESPIRATORY PANEL IN A MODULAR PCR SYSTEM

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SUMMARY

Avian respiratory pathogens are a leading cause of economic loss in the poultry industry. It can be difficult to identify infectious bronchitis virus (IBV), avian metapneumovirus (AMPV), infectious laryngotracheitis virus (ILTV), and paramyxovirus (PMV-1) as they can appear similar at different stages of infection. This PCR modular system can provide a preferred alternative to the currently available methods of identification, viral isolation, and serology. While these previous approaches were important for titer monitoring, they can be very time consuming and labor-intensive.

The RealPCR Avian Respiratory Panel was evaluated and demonstrated rapid identification with high sensitivity (< 10 copies per reaction) and included designs for specific North American IBV genotypes. The panel included IBV, ILTV, PMV-1, and AMPV (subtypes A and B) with specific genotype identification for IBV Ark, Mass, GA07, GA08, and DE/GA98. The IBV type specific tests allow quick analysis of genotypes circulating in the field which can be important for accurate vaccination decisions.

The RealPCR modular PCR platform allows simultaneous testing for each of these specified viruses, on the same plate. The shared reagents and standardized testing available with this platform will increase efficiencies and decrease testing and turnaround time for laboratories.

SPATIAL DISTRIBUTION OF FOUR PARASITIC HELMINTH SPECIES IN ONE, PEN-FREE, EGG-LAYING FACILITY AND THE CORRESPONDING EFFICACY OF NUTRACEUTICAL AND PHARMACEUTICAL ADMINISTRATIONS

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ABSTRACT

In the spring of 2019, adult (75 weeks old) brown laying hens from a commercial, pen-free, egg-laying facility (11,000 birds per house) located in Northwest Arkansas were obtained for the purposes of sourcing robust and consistent cestode infections for anticipated anthelmintic evaluations. To that end, 4 birds from each of six discrete sites (northwest, northcentral, northeast, southwest, southcentral, southeast) in one production barn were obtained on two occasions, 10 days apart, and necropsied for helminth counts. A definite, repeated, location to location variation in infection incidence and magnitude was seen for each of the two cestode parasite species present, and one of the two nematode parasite species present. Burdens of *Ascaridia galli* were generally similar regardless of bird location, with site-specific mean totals per bird over both sampling days between 31 and 80. For the remaining helminths, infections were greatest for birds from the southern half of the building as opposed to the northern, and from the western end of the barn as opposed to the central or eastern portions. Location specific, mean worm burdens over both sample dates ranged from 340 to 1133 (*Heterakis gallinarum*), 14 to 277 (*Raillietina cesticillus*), and 1 to 35 (*Choanataenia infundibulum*). Highest, individual bird infections were 299 (*A. galli*), 3575 (*H. gallinarum*), 1015 (*R. cesticillus*), and 102 (*C. infundibulum*). The above counts are for all developmental stages combined (*A. galli* and *H. gallinarum*) and for scolexes only (*R.* cesticillus and *C. infundibulum*); as determined via standard collection and quantification procedures utilizing both intestinal contents and overnight soaks.

Immediately prior to the mapping study outlined above, a set of birds was obtained and ultimately used according to IACUC oversight for the anthelmintic evaluation of fenbendazole in the water (5 mg/Kg BW for one day), levamisole in the water (8 mg/Kg BW for each of two days), herbal mixture in the feed (1 gm/4.5 Kg BW each day for 5 days), diatomaceous earth (2% of total feed for 10 days) and a nutraceutical mixture feed supplement (2% of feed for 7 days). Control trial efficacies for *Ascaridia galli* and *Heterakis gallinarum* were 0 and 12% for the nutraceutical feed additive, 0 and 22% for the diatomaceous earth feed additive, 0 and 26% for dietary herbals, 87 and 63% for levamisole, and 82 and 84% for fenbendazole, respectively.

Future studies are planned for:

1. Determining helminth infection patterns during bird tenure in a production house.
2. Anthelmintic efficacy evaluations for both nutriceutical and chemical interventions.
3. Enteric helminth-microbiome correlations.
4. Worm impact on feed efficiency and bird health.

INTRODUCTION

The initial, circumscribed objective of this line of research was to identify a flock of birds with a high incidence of cestode infections, and obtain a group of said birds for a cestocide (fenbendazole) efficacy study. Unforeseen findings in these pursuits necessitated the "shelving" of the planned cestocide study and instead undertake:

1. The mapping of helminth infections in a long-standing flock of birds.
2. Elucidate possible correlations between microbiome and gut helminth populations in the birds.
3. An efficacy study with various anti-helminth, commercial products that are being used for worm "control."

Data presented herein relate to items 1 and 3 above.

MATERIALS AND METHODS

Birds. All birds obtained for this study originated from a brown-egg, pen-free, operation in northcentral Arkansas. Birds at this operation were originally raised to 11 weeks of age at a remote pullet operation and then moved to the production unit for eventual depopulation at 75 weeks of age. The birds used in the mapping and efficacy studies were

approximately 75 weeks of age at the time of examination and experimentation. Production barns at this particular operation are approximately 40' by 380' and house 11,000 birds per barn at full capacity. Litter is replaced between each population of birds, and no anthelmintics are used.

Bird acquisitions. Originally, "representative" birds were obtained from the southwest corner of the barn and necropsied for worm burden determinations. After it was determined that this, single-point sampling of birds failed to elucidate the helminth burdens across the flock, birds were subsequently obtained for necropsy at 6 entry points; west, central and east points for both the south and north halves of the barn. The sampling was done twice in a 10-day period, with four birds obtained at each entry point per sample date.

Bird housing for anthelmintic evaluation. Birds discovered to have substantial populations of *Ascaridia galli* and *Heterakis gallinarum*, but basically "free" of tapeworm infections (not suitable for a planned cestocide study), were used in a nematocide study instead. To that end, the birds were randomly captured at the source farm and placed 10 per pen (10' by 12' pens, new bedding, central water plassons and canister feeders) in the bird housing unit at the University of Arkansas Parasitology farm. Acclimation was for 14 days prior to the start of experimental treatments.

Birds were maintained and utilized under approved IACUC procedures for the duration of their tenure at the unit.

Treatments. Six treatment groups were established on a per pen basis, with one pen of ten randomly-selected birds per treatment group. Ten control / untreated birds were posted at the start of treatments (day 0), and again at the end of all post-treatment periods (day 10). The treatments under investigation for nematocidal activity were:

Fenbendazole (Safe-guard®Merck), in the drinking water at 5 mg/kg BW for one day.

Levamisole (Prohibit®AgriLabs), in the drinking water at 8 mg/kg BW per day, for two consecutive days.

Strike III (®Durvet), in the feed at 2% concentration for *ad libitum* consumption for seven continuous days. This pelleted feed additive is a mixture of yeast culture, pumpkin seed, wormwood, clove, oregano, garlic, montmorillonite clay and diatomaceous earth.

J2160 Guan Zhong San herbal mixture, in the feed at the rate of 1 gm/ 4.5 kg BW/d for five consecutive days. This powdered, herbal feed additive has the listed ingredients of dryopteris, quisqualis, areca, melia, stemona, and carpesium.

Diatomaceous Earth (Earthsafe organics®Carl Pool), in the feed at a 2% concentration for *ad libitum* consumption for seven continuous days.

All medicated water and feed was observed to be ingested in total during the medication periods, with no adverse or untoward effects noted.

Parasite isolations. All helminth collections and quantifications were done according to the poultry guidelines as published by the World Association for the Advancement of Veterinary Parasitology (1), and as detailed in the University IACUC protocol specifically written for this work. To briefly summarize;

1 Bird euthanized by cervical dislocation.

2. Intestine and ceca removed, opened lengthwise, and all contents with wash are collected.

3. Tract soaked in water overnight under refrigeration and then the washing is collected.

4. All contents and washings rinsed over appropriate sieves and residues collected for parasite retrieval under stereoscopic microscopy, identification and counting.

RESULTS

A. Helminth infection "mapping." On two occasions, 10 days apart, four birds were taken from each of six designated points of entry into one poultry house (75 weeks old, brown hens). Excellent consistency of worm incidence and numbers was seen between the two dates at each entry point. Mean worm numbers over both dates are presented in Table 1; summarized north half versus south half, and east versus center versus west thirds. For all four helminths isolated and identified in these birds (*Ascaridia galli*, *Heterakis gallinarum*, *Raillietina cesticillus*, and *Choanataenia infundibulum*), birds from the southern half of the barn averaged twice as many parasites as birds from the northern half. It was also seen, that with the exception of *A. galli*, birds from the western third of the house were the most parasitized.

For all birds over both sample dates, 100% were infected with *A. galli* and *H. gallinarum*, 69% were infected with *R. cesticillus*, and 36% were infected with *C. infundibulum* (Table 2). In the same order, highest single bird worm infections were 299, 3575, 1015 and 102. Mean stage of development numbers for the nematodes are presented in Table 3.

B. Efficacy study. Only adult nematode burdens were used for efficacy evaluations as only their populations were consistent enough for any sort of meaningful interpretation of results. Per treatment group, mean *A. galli* and *H. gallinarum* burdens are presented in Table 4. Percent efficacies for adult nematode populations, as obtained from Table 4

numbers, are presented in Table 5. To be "technically" considered efficacious, an anthelmintic should remove > 90% of a targeted parasite population; a level of efficacy that was not displayed by any product. However, the chemical anthelmintics (fenbendazole and levamisole) displayed much greater efficacy than any of the "non-chemical" products (strike, herbals and diatomaceous earth).

DISCUSSION

This report concerns two aspects of helminth infections acquired and maintained by commercial laying hens housed in pen-free, indoor systems: the establishment of long-standing helminth burdens and effectiveness of anthelmintic intervention. Relative to the infections occurring naturally in the birds, it was shown that bird infections by A. galli appear to be relatively uniform across a house. Infections by H. gallinarum, R. cesticillus and C. infundibulum were extremely varied relative to bird location within the house (north versus south, east versus middle versus west); a difference that was observed at two sample times. This observation spurs two considerations; (1), that random sampling across the whole bird population must be employed if a true estimation of worm burdens is to be obtained for an entire flock of birds, and (2), diverse, in-house populations of helminths become established and maintained, even though replacement birds are brought in as a homogeneous population from pullet operations with evenly dispersed parasitisms amongst the birds at the time of placement.

Relative to anthelmintic intervention, it was shown that feeding "natural" products (herbals, diatomaceous earth and diatomaceous earth supplemented with additional natural products) resulted in no decrease in adult A. galli burdens, but perhaps a slight decrease in cecal worm burdens (H. gallinarum). Chemical intervention (levamisole and fenbendazole) did result in "good" reductions for both cecal worm and roundworm populations; albeit not at the "efficacious" levels of > 90%. Additional investigations relative to the effectiveness of anthelmintic-type products is warranted regarding the parasitisms studied herein, as the current results might be site-specific, worm burdens were too varied to gain real statistical inferences, and products might be given at different rates and durations.

The impact of helminth parasitisms in farm animals, poultry included, is for the most part subclinical (2). True economic loss incurred by the producer is "hidden" as anorexia, lowered feed efficiency and lowered production. These losses can be measured with controlled studies, but rarely, if ever, seen in the field. Given the levels of helminthiasis seen in the current study, with individual birds having as many as 299 roundworms, 3575 cecal worms and 1117 tapeworms, considerable economic loss was certainly ongoing; a scenario which deserves additional investigation relative to worm impact and control. Researchers have recently shown that feed conversion and egg production are significantly, negatively impacted when worm burdens were approximately half of what is reported here (3). Additionally, as no anti-helminth measure tested in the efficacy study proved "efficacious" (> 90% effective), concern is deserved here as well as these products are routinely used for worm "control". It should be noted however that fenbendazole was not given at the recommended regime (1 mg/kg BW / day for 5 consecutive days, but rather as 5 mg/kg BW on one day). All other products were given per label directions.

As a follow-up to the work presented here, we are currently looking into more anthelmintic studies, microbiome-worm correlations, worm impact on feed efficiency and worm in-barn, on-farm and between farm variances in population dynamics.

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Table 1. Arithmetic means for individual bird, total helminth burdens by house location over both sampling dates.

Helminth	House Location				
	South	North	East	Center	West
A. galli	54.7	30.6	38.9	57.4	31.6
H. gallinarum	890.3	428.3	434.3	687.6	855.9
R. cesticillus	107.2	54.5	32.2	33.9	176.4
C. infundibulum	17.8	6.7	7.9	5.7	23.1

Table 2. Helminth incidence and individual bird, helminth ranges for birds obtained over both sampling dates and all house locations.

Helminth	Percent	
	Incidence (%)	Range
A. galli	100	1-299
H. gallinarum	100	13-3575
R. cesticillus	69	0-1015
C. infundibulum	36	0-102

Table 3. *A. galli* and *H. gallinarum* burden means by developmental stage.

Helminth/stage	Mean
A. galli:	
L2	3.3
L3	9.4
L4	3.9
adult	26.1
H. gallinarum	
larval	336.8
adult	322.4

Table 4. Arithmetic mean, helminth burdens by treatment group at necropsy.

(SD)

Treatment	Helminth and Stage					
	A. galli			*H. gallinarum*		
	Larval+	Adult	Total	Larval++	Adult	Total
Fenbendazole	2.1 (2.0)	0.7 (0.9)	2.8 (2.7)	0.3 (0.7)	12.6 (20.1)	12.9 (20.0)
Levamisole	7.4 (10.7)	0.5 (1.0)	7.9 (10.6)	0.0 (0)	30.0 (50.2)	30.0 (50.2)
Strike	5.8 (8.2)	7.1 (7.0)	12.9 (11.1)	2.5 (6.9)	72.1 (105.7)	74.6 (105.4)
Herbal	1.0 (1.5)	4.1 (4.4)	5.1 (5.0)	2.9 (7.1)	60.3 (57.2)	63.2 (62.3)
Dia. earth	9.1(16.9)	5.7 (8.3)	14.8 (18.7)	0.3 (0.5)	63.5 (64.6)	63.8 (65.0)
Day 0 control	12.6 (20.1)	5.4 (6.4)	18.0 (22.9)	5.7 (8.4)	156.6 (147.5)	162.3 (145.4)
Day 10 control	3.3 (6.3)	3.9 (3.2)	7.2 (6.7)	5.0 (8.3)	81.8 (94.2)	86.8 (95.9)

+ L3 and L4 stages combined
++ all larval stages combined

Table 5. Percent reductions in adult helminth populations compared to day 10 control birds.

Treatment	Helminth	
	A. galli	*H. gallinarum*
Fenbendazole	82.1	84.6
Levamisole	87.2	63.3
Strike	0.0	11.9
Herbal	0.0	25.3
Dia. earth	0.0	22.4