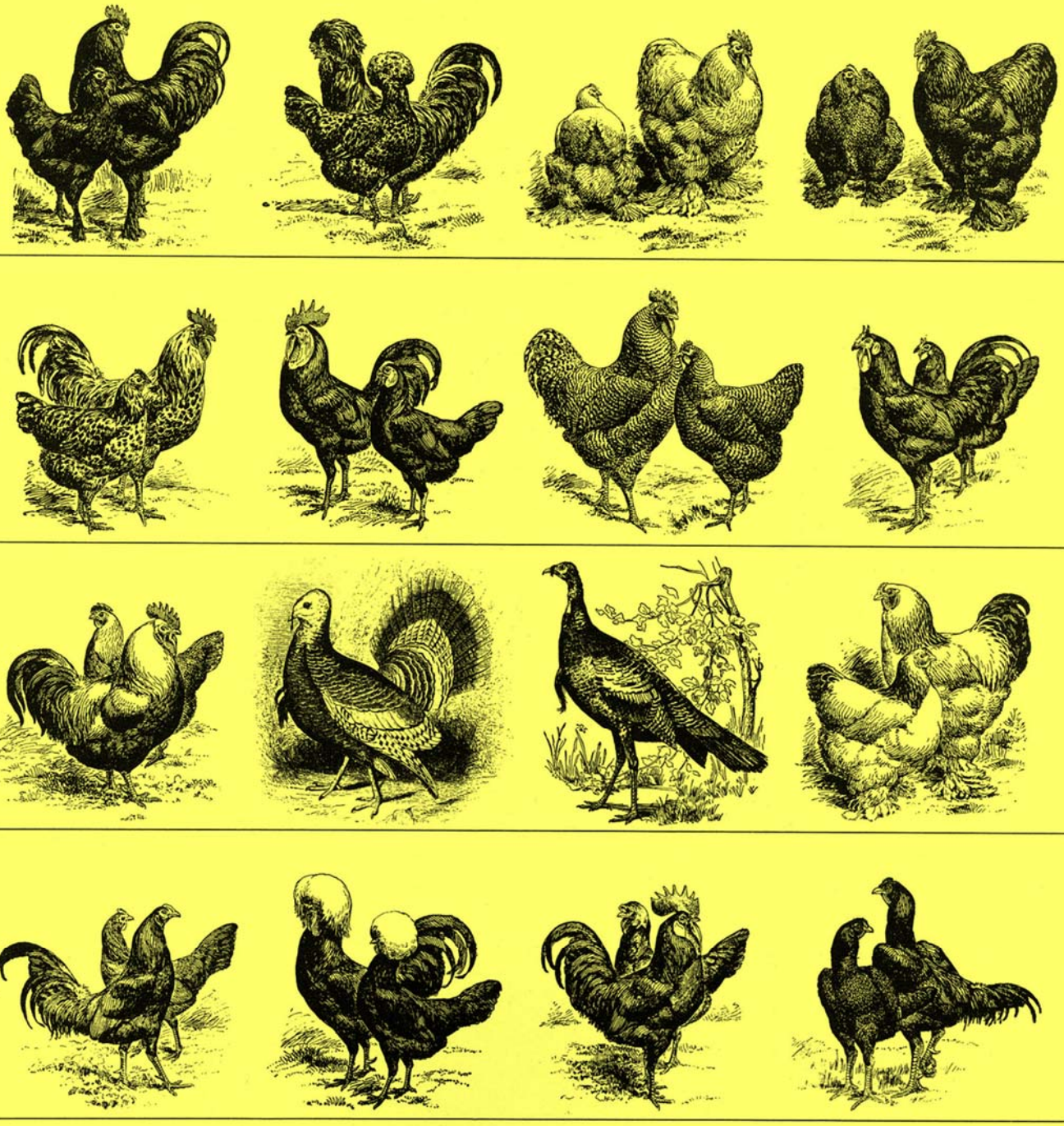
PROCEEDINGS OF THE SIXTY-SIXTH WESTERN POULTRY DISEASE CONFERENCE

March 20-22, 2017 Sacramento, CA



PROCEEDINGS OF THE SIXTY-SIXTH WESTERN POULTRY DISEASE CONFERENCE

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66TH WESTERN POULTRY DISEASE CONFERENCE DEDICATION

RICHARD McCAPES

Richard "Dick" McCapes was born in 1933. He attended the University of California, Davis (UCD), School of Veterinary Medicine (SVM), and graduated in 1958 with his DVM. While at UCD, Dick met his wife, Marilyn, and they were married in 1955. Following graduation, Dick went into private practice in the San Luis Obispo area. However, in 1970, the McCapes and their children moved back to Davis where Dick was hired as a lecturer in the UC Davis SVM.

Since 1970, Dick has held a number of positions in the vet school, including serving as Chair of the Department of Epidemiology and Preventive Medicine. Most of his research has been carried out in cooperation with laboratory-based faculty on a variety of poultry health problems, including investigations involving mycoplasma infections, salmonellosis, paramyxovirus type 3, hemorrhagic enteritis, lymphoproliferative disease of turkeys, and avian influenza.

Perhaps Dr. McCapes' hallmark accomplishment was the development and direction of the SVM's Avian Medicine Residency Program. This offering was designed to provide a level of advanced training in clinical avian medicine that would enable the graduates to function effectively in a poultry industry-related corporate environment, in a private practice, or in an academic setting. This program had no equal and has been proven to be highly successful. (The first resident was Dr. Gregg Cutler in 1976.)

Dr. McCapes served as President of the AAAP in 1978-79, and actively supported the WPDC, serving as Program Chair (1968) and President (1969).

The McCapes have experienced almost every possible aspect of UC Davis. In more than six decades as UCD Aggies, they have been students, alumni, faculty, staff, retirees, volunteers and alumni Life Members. Dick, and his late wife Marilyn, were recently honored at the Cal Aggie Alumni Association's Award Gala, which took place on February 3, 2017 at UCD, by receiving the 2017 Aggie Service Award in recognition of their exemplary Aggie pride and dedication to UC Davis.

It is with great appreciation and honor that the 66th Western Poultry Disease Conference be dedicated to Dr. Richard McCapes.

66th WPDC SPECIAL RECOGNITION AWARD
PETER WOOLCOCK

Peter was born in 1944 and raised in post-war Central London. He graduated from Birmingham University with a degree in botany, but had gained an interest in microbiology. This resulted in obtaining his MSc in general virology the following year. Subsequently, Peter attended Leeds University and graduated in 1974 with a PhD in microbiology. Peter met his bride-to-be, Lesley, while both were attending technical college in London. They were married in London in 1968, and had two boys, Chris and Rob, both born in Bury St. Edmunds, Suffolk.

Peter's first job as a virologist was working at the Animal Health Trust near Newmarket in East Anglia. Here, he worked on duck hepatitis virus (DHV) and other duck diseases, including *Chlamydia psittaci*; however, in 1984 funding for his research ended. Peter's next professional opportunity came in 1985 when he received a call from Bruce Calnek at the Cornell Duck Research Laboratory on Long Island, NY. So, in 1986, at the age of 41, Peter and Lesley packed up their belongings, and with their two boys headed west, across the pond, to the USA and their "American Dream."

While at the Duck Research Laboratory, Peter continued his work with DHV. He developed a plaque assay in cell culture which allowed them to make an inactivated vaccine that could be tested by monitoring the immune response in vaccinated ducks by assaying for neutralizing antibody in the plaque assay in cell culture. He also produced DHV and DVE vaccines and bacterins for *Riemerella anatipestifer* and *E. coli*.

However, once again, funding became extremely tight, and in 1991 Peter found himself looking for his next great opportunity. That came in the form of a phone call from a colleague, H.L. Shivaprasad, aka, Prasad, who was previously at Cornell University at the same time Peter was there. The UC Davis, School of Veterinary Medicine had recently taken over administration of the California Animal Health and Food Safety Laboratory System (CAHFS) and was looking for an avian virologist for the system, who would be located in Fresno, CA. Once again, Peter, Lesley and the boys packed their bags and headed west to sunny California, the "land of fruits and nuts!"

For the next 18 years, Peter flourished as the avian virologist for CAHFS. He recalls numerous exciting cases, including the NDV outbreak in 2002, identifying Hepatitis E virus, isolation of very virulent IBDV, isolation of WNV, and many isolations of AIV. During this time, he was author or co-author on almost 70 publications in refereed journals, 35 book chapters, and numerous presentations and abstracts. He was a member of the editorial committee for the 5th and 6th editions of "A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens."

Once again, however, times got tough and the Fresno lab was closed in 2009 due to a budget crisis in California. Peter was relocated to the CAHFS-Davis laboratory, where he continued his work as the laboratory system's avian virologist until his retirement in 2013.

Peter and Lesley continue to live in Fresno. Peter now spends his time swimming, gardening, studying photography, doing yoga, and traveling. Peter has also recently gotten involved with local government by serving as a trustee on the Fresno Mosquito and Vector Control District Board. They visit their sons and grandchildren as often as possible. One son lives on Long Island, NY, and the other son, along with his wife and Peter's two grandchildren, live in Houston, TX.

The 66th WPDC is honored to recognize Dr. Peter Woolcock for his service to the advancement of knowledge of avian diseases in the western region.

WESTERN POULTRY DISEASE CONFERENCE
DISTINGUISHED SERVICE AWARD

RICHARD P. CHIN

After many dedicated years involved with the Western Poultry Disease Conference (WPDC) in many capacities, Rich is relinquishing his crucial spot on the WPDC Executive Committee roost (or so he says).

Dr. Richard Chin was born October 26, 1957 and was raised in San Mateo, California. He received a B.S. degree in Animal Science (1979), Doctor of Veterinary Medicine degree (1983), and Master's degree in Preventive Veterinary Medicine (1985) from the University of California, Davis. In 1986 he completed a residency program in Avian Medicine at the Turlock laboratory. During his subsequent career with the California Veterinary Diagnostic Laboratory System and California Animal Health & Food Safety Laboratory System, Rich served as Branch Chief of the Fresno Branch Laboratory before being transferred to the Tulare lab because of the Fresno lab closure. He officially retired in 2015, finishing his career in Tulare. He and his wife Elaine have recently moved into a newly-built house in Petaluma.

Over the course of his career, Dr. Chin has been the author or co-author of at least 48 peer-reviewed refereed publications and has been contributing author for chapter sections addressing *Ornithobacterium rhinotracheale* and *Mycoplasma meleagridis* in "Diseases of Poultry."

Rich has always considered professional service as an important component of his career. He served on committees, on the board of directors (1996-2000), and as president (2004-05) of the American Association of Avian Pathologists. Dr. Chin was the recipient of the Lasher-Bottorff Award in 2012. He has also given valuable service to the American Veterinary Medical Association and as a long-time member of the Technical Committee of the National Poultry Improvement Plan. Just as significantly, Dr. Chin has participated in important advisory capacities assisting the poultry industries within California.

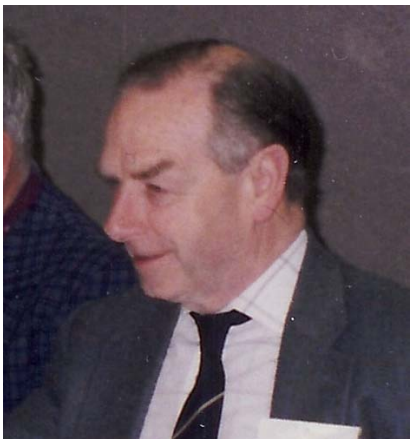
Rich first began attending the WPDC in the mid-80s when it was still being held at the UC Davis Faculty Club. Over the next ten years or so, he regularly presented papers and "learned the ropes" of the organization. In 1991 he served as program chair of the joint 40th WPDC/XVI ANECA meeting held in Acapulco, Guerrero, Mexico. This was a difficult organizational undertaking at the time, and Rich completed his task admirably. The following year he served as president of the WPDC in Sacramento.

Over the next few years, changes needed to occur regarding the WPDC organizational roles and Rich worked closely with Rosy and others during this period. In 1997, Rich officially assumed the position of secretary-treasurer – position he has held for 20 years. It is significant to note that he has not missed a WPDC since attending his very first meeting. It is impossible to enumerate the number of lives he has touched in positive ways and people he has guided and helped during those years.

Therefore, it is with great pride and pleasure that the Western Poultry Disease Conference Distinguished Service Award be presented to Dr. Richard Chin for his many years of dedicated time and service to all of us involved in the WPDC. We look forward to many more years working with Rich as a valued WPDC adviser, and wish him well in his "retirement."

IN MEMORIUM

DUNCAN McMARTIN



Dr. Duncan McMartin, of Davis, California and Rannoch, Scotland, passed away at home on Jan. 14th, 2017. McMartin was a member of the school's faculty from 1980-1993 in our Vet Extension Unit and in the Department of Population Health and Reproduction. McMartin provided leadership and advanced scientific knowledge of avian health problems. His research focused on *Salmonella* Enteritidis. He later took a leadership role in developing the Veterinary Extension Animal Welfare Program to promote broad dialogue and understanding related to human-animal inter-relationships, and societal concerns related to animal welfare in research, education and animal agriculture.

He was a well-respected veterinarian, and an accomplished fiddler player. He began playing the fiddle as a young boy in Scotland, but higher education and his veterinary career took him away for more than four decades. Upon retiring in 1994, he resumed his interest in Celtic fiddle music and joined a group to continue his passion. He was invited to perform at the Spring Faculty Reception in 1997.

Duncan was a frequent attender of the Western Poultry Disease Conference and served as the program chair for the 35th WPDC/XI ANECA combined meeting and president of the 36th WPDC. He received the WPDC Special Recognition Award in 1998.

SPECIAL ACKNOWLEDGEMENTS

The 66th 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. More than 40 organizations, companies and individuals have once again given substantial financial support. Many companies and organizations, including some that also contribute financially, 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 following pages. We greatly appreciate their generosity and sincerely thank them and their representatives for supporting this year's meeting of WPDC.

Dr. Gabriel Sentíes-Cué, Program Chair of the 66th WPDC, would like to thank his wife and family for supporting him all these years, especially since coming to the USA. Additionally, he thanks CAHFS and CDFA for the opportunities and support they have provided him in the growth of his career. Dr. Sentíes-Cué is thankful to all the presenters and invited speakers for their participation, and to Dr. Rich Chin and Dr. David Frame for their suggestions and help in developing the scientific program. Finally, a sincere thank you to Ms. Jamie Nunes for her outstanding assistance.

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 for her exceptional work with our conference. In addition, Dr. Chin would like to thank Ms. Elvie Martins, Ms. Aundrea Turner and the California Animal Health & Food Safety Laboratory System, for their continual administrative support, and Bob and Janece Bevans-Kerr 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.

66th WPDC CONTRIBUTORS LIST

(As of March 15, 2017)

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66th WPDC PROCEEDINGS

Please note that the proceedings of the 66th 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 66th WPDC are available in electronic format only. They can be downloaded from the American College of Poultry Veterinarians website (www.acpv.info).

WESTERN POULTRY DISEASE CONFERENCE (WPDC) HISTORY

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

YEAR	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
35th WPDC – 1986	D. W. Waldrip	Duncan A. McMartin	J. A. Allen	
11th ANECA	Jorge Basurto	Mario Padron	A. Tellez-G. Rode	
36th WPDC – 1987	D. A. McMartin	Marcus M. Jensen		
37th WPDC – 1988	M. M. Jensen	Barry Kelly	A. S. Rosenwald	
38th WPDC – 1989	B. Kelly	Masakazu Matsumoto		Louise Williams
39th WPDC – 1990	M. Matsumoto	Jeanne M. Smith		Dean Young
40th WPDC – 1991	J. M. Smith	Richard P. Chin	A. S. Rosenwald	
16th ANECA	Martha Silva M.	David Sarfati M.	A. S. Rosenwald	
41st WPDC – 1992	R. P. Chin	Rocky J. Terry	Marcus Jensen	Henry E. Adler *
				*(posthumous)
				R. A. Bankowski
				C. E. Whiteman
42nd WPDC – 1993	R. J. Terry	A. S. Dhillon	W. W. Sadler	Royal A. Bagley
43rd WPDC – 1994	A. S. Dhillon	Hugo A. Medina		G. B. E. West
44th WPDC – 1995	H. A. Medina	David D. Frame	W. M. Dungan*	A. J. DaMassa
			*(posthumous)	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*	B. Daft
			*(posthumous)	
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

MINUTES OF THE 65TH WPDC ANNUAL BUSINESS MEETING

President Dr. Shahbaz Haq, called the meeting to order on Monday, April 25, 2016, at approximately 4:20 PM, at the Vancouver Marriott Downtown Hotel. There were 16 people in attendance.

APPROVAL OF 64th WPDC BUSINESS MEETING MINUTES

The minutes from the 64th WPDC business meeting were discussed. Since a hardcopy of the proceedings was not produced, members of the Executive Committee reviewed the minutes during the Executive Committee meeting and recommended approval as written. A motion was made and carried to approve the minutes as recorded in the Proceedings of the 65th WPDC.

ANNOUNCEMENTS

Dr. Chin acknowledged all the contributors, in particular, Ceva Animal Health, which contributed at the Super Sponsor level, and the American Association of Avian Pathologists, which contributed at the Benefactor level. All the contributors were acknowledged and thanked for their generous support and donations.

The efforts of the current WPDC officers were acknowledged for their work and participation in the organization of this year's meeting.

We remembered John Voris who passed away since the last WPDC. John worked for Nicholas Turkey Breeding Farms and the University of California Extension Service as a specialist in turkeys.

REPORT OF THE SECRETARY-TREASURER

Dr. Chin presented the Secretary-Treasurer report. For the 2015 meeting, there was a net profit of $3,672.27. As mentioned last year in the Business meeting, we had $29,450 in contributions.

Unfortunately, Dr. Chin estimates that there will be a net loss this year. Contributions should be slightly higher ($30,825) if all pledged contributions are given. Nonetheless, he estimates a net loss due to increases in travel, hotel (food and guest rooms), and the purchase of two laptop computers for use by those in the Executive committee and presentations at the meeting.

REPORT OF THE PROCEEDINGS EDITOR

Dr. David Frame presented the Proceedings Editor report. There were 109 papers submitted for publication in the proceedings. He thanked the authors for their timely submissions.

WPDC continues to be grateful to the American College of Poultry Veterinarians for providing space on their website to host the WPDC proceedings. As approved last time, all WPDC proceedings on the ACPV website are not password-protected, but free-of-charge.

FUTURE MEETINGS

It was agreed to continue with the current rotation for meeting venues, with three different locations, i.e., Mexico, Canada, and a location yet to be determined. WPDC will continue to return to Sacramento every other year. The following schedule was tentatively set:

2018: 67th WPDC, Salt Lake City, April 15-18, 2018
2019: 68th WPDC, Sacramento, CA
2020: 69th WPDC, Mexico
2021: 70th WPDC, Sacramento, CA
2022: 71st WPDC, Canada
2023: 72nd WPDC, Sacramento, CA

People were reminded that they vote on the locations each year, so it can be changed.

WPDC EXECUTIVE COMMITTEE

Dr. Chin reported that the WPDC Executive Committee nominated Dr. Rodrigo Gallardo for Program Chair for the 67th WPDC in 2018 (in Salt Lake City, UT). There were no other nominations and Dr. Gallardo was elected unanimously as program chair-elect. The following officers were nominated for 2016-2017:

Program Chair: Dr. Gabriel Senties- Cué
President: Dr. Susantha Gomis
Past-President: Dr. Shahbaz Haq
Contributions Chair: Dr. Yan Ghazikhanian
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Richard Chin
Program Chair-elect: Dr. Rodrigo Gallardo

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

NEW BUSINESS

Dr. Chin stated the Dr. Gallardo has agreed to take over as WPDC Secretary-Treasurer in July 2018 – the same time when he will be the WPDC President. The group thanked Dr. Gallardo for accepting the position.

Dr. Chin stated that CE credits will be sent to every registrant from ACPV (Bob Bevans-Kerr). There were no additional items for discussion.

Dr. Haq turned the presidency over to Dr. Gomis who acknowledged and thanked those who helped organize this year's meeting.

Dr. Gomis adjourned the annual business meeting at 4:50 PM.

THE ARNOLD S. ROSENWALD LECTURE

Carol J. Cardona
2017

THE PRECARIOUS BALANCE BETWEEN DISEASE PREVENTION AND POULTRY PRODUCTION

C. J. Cardona, Katharine L. Schlist, Rebecca A. Johnson, and D. A. Halvorson

Arnold "Rosy" Rosenwald's career as an Extension Poultry Pathologist embodied the role that cooperative extension can play in agricultural production. There are many milestones and achievements that marked his career but perhaps none more than the work he did in defining the role of universities and extension in a foreign animal disease (FAD) outbreak. Rosy and Art Bickford, the legendary poultry pathologist and Rosy's successor as an extension veterinarian, were pioneers in creating an open dialogue with industry and regulatory authorities about the velogenic Newcastle disease outbreak that changed the face of the California poultry industry in the 1970s. They confronted practices done in the name of disease control and looked for evidence of effectiveness. Although it may have seemed like no one was listening at the time, Rosy's and Art's work left a legacy for academics that was followed again for the 1983 Pennsylvania HPAI outbreak, the exotic Newcastle disease outbreak of 2002, and again in the 2014-5 HPAI outbreak. Throughout his life Rosy spoke to evidence and advanced truth, and often did so through the Western Poultry Disease Conference and its proceedings where stories of poultry health and disease were and are told. We hope that this record of the 2015 Midwestern US highly pathogenic avian influenza (HPAI) outbreak story continues Rosy's legacy.

DESCRIPTION

Summary of the 2015 Midwestern US HPAI outbreak. On Friday February 27, 2015 rapidly increasing mortality was observed in a flock of turkey breeder hens. By Thursday March 5 an official diagnosis of HPAI H5N2 was announced. Thus began an outbreak that lasted for three months and resulted in the infection of millions of turkeys and egg laying chickens. No broiler flocks were infected although some were affected by the outbreak. Additionally, although birds with outdoor access like backyard flocks and gamebird farms are frequently blamed for disease outbreaks, few flocks managed this way were infected in this outbreak. The last infected flock in this outbreak was detected on June 16, 2015 and the outbreak was declared over on November 13, 2015 (2).

Sources of information on the outbreak and what they tell us. USDA APHIS VS conducted extensive epidemiological analyses of the HPAI outbreak; most noteworthy were the investigation of possible airborne transmission of the virus, an egg layer case-control study, and molecular analysis of virus isolates from the outbreak. Investigators looked for evidence of airborne transmission by multiple methods but were unable to determine whether aerosol transmission was responsible for a farm becoming infected or not (1, 7). In the case-control study of egg layers, epidemiologists were able to demonstrate that being in a control zone, rendering trucks or garbage trucks going near barns, and visits by service personnel accounted for 89% of the average attributable fraction of cases (4); whereas a hard surface pad at the barn entry and changes of footwear and clothing prior to barn entry were protective. Although there were epidemiological surveys (i.e., case series) of some of the affected turkey farms (3), no case-control study involving turkeys has been published to date. A phylogenetic analysis of virus isolates (eight

genes) indicated that the Midwest was affected by multiple point source or independent introductions as well as common source or lateral spread (1).

Utilizing this information, and incorporating conversations with industry and regulatory veterinarians who lived through the outbreak, it became clear that the outbreak was largely spread through activities that are part of normal production practice. And, while these activities don't spread disease under usual conditions, they are likely to spread an FAD. This is because an FAD is anything but routine.

Piecing together how the outbreak spread. Clearly, the biosecurity that was in place during the 2015 outbreak was insufficient to prevent cases of H5N2 HPAI. Biosecurity in the poultry industry is largely designed to prevent routine, endemic disease agents on one premises from entering another premises, or from being transferred between successive flocks on a single farm. These endemic threats are a routine economic burden that can be lessened with biosecurity often including vaccination. Because these disease agents are common, systems and practices are in place to prevent their spread and are economically and practically feasible. Host immunity is a key to not only preventing the consequences of disease but also in reducing viral shedding by infected hosts so that routine levels of biosecurity are effective (6). This is in marked contrast to an exotic disease agent where the absence of host immunity means extremely high levels of the pathogen are shed during infection. The amount of pathogen shed when fully susceptible hosts are infected vs. when immune, partially immune, or chronically infected flocks are infected is much larger and results in a far greater challenge to the biosecurity of nearby premises with susceptible poultry. Environmental sampling for HPAI virus and testing by RRT-PCR during the 2015 outbreak showed approximately three logs more virus on heavily contaminated premises (those which had delayed depopulations and thus many, many infected birds) compared to less contaminated premises (those with rapid depopulation and thus many fewer infected birds) (5). Therefore, one of the problems in dealing with a FAD is that the operational and structural biosecurity levels that are good enough to prevent endemic disease transmission are insufficient to prevent the transmission of an FAD. In the end, people thought they had the biosecurity they needed but the challenge was far greater than they and their prevention strategies were ready to meet.

Part and parcel to having insufficient levels of biosecurity in place to prevent infections in the face of incredibly high viral loads, there also were practices that continued during the outbreak that increased the vulnerability of poultry farms. The concepts we discovered were not unique to this outbreak, and we wondered how poultry companies could continue to use these practices which had time and again been linked to the transmission of disease from farm to farm. One example of this is the movement of dead birds off-site for disposal, a practice which results in a high potential for the movement of disease agents. In exploring the practices more closely, we found that many of these activities play an essential role in production and the current practice is the most cost effective. For these reasons, such activities cannot be eliminated and replaced with other practices or approaches in a sustainable way. Thus, the risks of disease transmission associated with these types of practices must be mitigated through the varied application of traffic flow separations and sanitation or discontinued altogether, usually at a cost, during an outbreak.

Other practices that were identified could be commonly linked to labor shortages or human resource cost savings. Specifically, sharing employees among farms is common for activities that require additional labor. For example, on pullet farms, although a few employees can easily care for the birds on a daily basis, they need help to vaccinate a flock. In such situations, outside/independent/contract crews typically are employed to do the work and these individuals are commonly shared between premises. This practice is always risky because people are very good fomites for disease agents. For endemic diseases, the risk can be successfully mitigated; however, with an FAD, the viral loads are far too great and the birds are far too susceptible for successful risk mitigation. The continuation of this practice during the outbreak likely resulted in new cases.

Not how but what spread the outbreak. The 2015 H5N2 Midwest outbreak was spread through the movement of virus from infected birds, their eggs, and their manure. These materials contaminated shared equipment that spread the virus to new populations via off-site mortality disposal (3, 4), garbage removal (4, 8), and other activities. In addition, these materials contaminated people that were shared among premises including: families employed at different poultry operations (3), service personnel (4), and outside/independent/contract crews (personal communication with poultry veterinarians). Everything and anything that could carry these materials, including the air (7), were implicated in spread of the virus.

Every ranch is different. Rosy always ended his newsletters with the letters "ERID" which means *every ranch is different.* Those words are probably truer during an outbreak than in normal times. That's because during an outbreak, new threats are being introduced every day, and managing risk requires real expertise and attention to the details of the individual farm. We noticed that in this outbreak there were fewer veterinarians employed in private industry than in previous outbreaks. As vaccines, management, and housing systems have been improved over the years, the incidence of disease has decreased on poultry farms. As a result, there has been a decrease in the number

of veterinary professionals per bird employed in private industry. We heard from poultry industry veterinarians and producers alike that the 2015 Midwest HPAI outbreak was larger and longer because there were not enough people with the expertise needed to address the real risks facing a specific farm.

CONCLUSION

The largest FAD outbreak in the history of the US was spread in the usual ways by the usual suspects. Many of the risky practices that contributed to spread were known *prior to the outbreak*. The practices that create biosecurity vulnerability continued during the outbreak and continue to be used today because they can't adequately be replaced in an industry structured the way this one is. Once the outbreak began, practices could not be changed or suspended rapidly enough to prevent the majority of the cases and there were not enough experts needed to address individual farm threats.

REFERENCES

1. Epidemiologic and Other Analyses of HPAI-Affected Poultry Flocks. In. B. McClusky, ed. USDA-APHIS-VS, www.aphis.usda.gov/animal_health/animal_dis_spec/poultry/downloads/Epidemiologic-Analysis-Sept-2015.pdf. p 102. September 9, 2015.

2. Clifford, J. Highly pathogenic avian influenza, United States of America. In. OIE, www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=19116. 2015.

3. Dargatz, D., A. Beam, S. Wainwright, and B. McCluskey. Case Series of Turkey Farms from the H5N2 Highly Pathogenic Avian Influenza Outbreak in the United States During 2015. Avian Dis 60:467-472. 2016.

4. Garber, L., K. Bjork, K. Patyk, T. Rawdon, M. Antognoli, A. Delgado, S. Ahola, and B. McCluskey. Factors Associated with Highly Pathogenic Avian Influenza H5N2 Infection on Table-Egg Layer Farms in the Midwestern United States, 2015. Avian Dis 60:460-466. 2016.

5. Lopez, K. N., J. Johnson, R. Halvorson, D. Jensen, J. Guo, X. Culhane, M. Flores-Figueroa, C. Muñoz-Aguayo, J. Cardona, C. Detection of H5N2 Highly Pathogenic Avian Influenza Virus on Infected Layer Farms in Minnesota and Iowa. In: Western Poultry Disease Conference. D. Frame, ed., Vancouver, BC. pp 151-154. 2016.

6. Sen, S., S. M. Shane, D. T. Scholl, M. E. Hugh-Jones, and J. M. Gillespie. Evaluation of alternative strategies to prevent Newcastle disease in Cambodia. Prev Vet Med 35:283-295. 1998.

7. Torremorell, M., C. Alonso, P. R. Davies, P. C. Raynor, D. Patnayak, M. Torchetti, and B. McCluskey. Investigation into the Airborne Dissemination of H5N2 Highly Pathogenic Avian Influenza Virus During the 2015 Spring Outbreaks in the Midwestern United States. Avian Dis 60:637-643. 2016.

8. Walz , E., E. Linskens, J. Umber, M. Culhane, D. Halvorson, F. Contadini, and C. Cardona. Risk of a Poultry Flock Becoming Infected with HPAI-virus due to garbage Management. In: Western Poultry Disease Conference. D. Frame, ed., Sacramento, CA. 2017.

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ENHANCED MACROPHAGE RESPONSE POST-HATCH IN CHICKENS FOLLOWING *IN OVO* DELIVERY OF NUCLEIC ACIDS

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SUMMARY

Nucleic acids such as single stranded ribonucleic acids (ssRNA) and oligonucleotides containing unmethylated CpG motifs (CpG-DNA) are recognized by toll-like receptor (TLR) 7 and 21 in chickens respectively and known to elicit protective responses against a number of poultry viral and bacterial diseases when delivered *in ovo*. However, the information on the mechanisms of protection are scarce. The objective of the study was to investigate the macrophage response in respiratory and gastrointestinal tracts of post-hatch chickens following *in ovo* delivery of ssRNA and CpG-DNA. We delivered ssRNA, CpG-DNA or PBS *in ovo* and collected tissues from the respiratory and gastrointestinal tracts for the purpose of macrophages immunostaining. We found that both nucleic acids are capable of eliciting strong innate immune responses characterized by macrophage increase in respiratory and gastrointestinal systems potentially implicating macrophages in the protective host responses against microbial infections in chickens.

INTRODUCTION

The innate immune system, which mounts potent, nonspecific and broadly effective host responses, is equipped with a range of immune cells. One of the key immune cells indispensable in this regard is the macrophages. It has been shown in many studies that the macrophages play major roles in host responses against a number of microbes (5-7, 13) through the recognition of broadly encoded, highly conserved microbial molecules known as pathogen-associated molecular patterns (PAMPs)(2). This recognition is mediated by the receptors expressed on macrophages as well as in other immune and nonimmune cells in the host known as PRRs (11). Toll-like receptors (TLRs) are the well-studied host receptors that are indispensable in recognizing PAMPs and eliciting appropriate host responses (9, 10).

Each TLR binds with a unique set of PAMPs of microbes in order to activate the signaling pathways. The recent advancement in the understanding of pathogen-TLR interaction has facilitated in developing synthetic ligands, which can be used therapeutically or prophylactically to stimulate innate host responses against various infectious diseases in mammals and birds. In such synthetic ligands, single-stranded ribonucleic acid (ssRNA) (3) which is a TLR7 ligand and Cytosine-guanosine deoxynucleotides (CpG DNA) which is a TLR21 ligand in avian species (4, 8) have become a research focus as an immunotherapeutic agents.

In ovo vaccination at embryo day (ED)18 has become a common practice in the poultry industry against many diseases (for example, Marek's disease) inducing early immunity in birds than post-hatch vaccination (12, 14, 16, 18, 19). Previously, it has been shown that many TLR ligands elicit protective host responses against a number of poultry viral and bacterial diseases when delivered *in ovo* (1, 15, 17, 18). However, the information on the mechanisms of protection are scarce. The objective of the study was to investigate the macrophage responses in respiratory and gastrointestinal tracts of post-hatch chickens following *in ovo* delivery of ssRNA and CpG-DNA.

MATERIALS AND METHODS

In this study, we delivered ssRNA (100µg) or CpG-DNA (50µg) *in ovo* at ED 18 with a control group receiving PBS. At day one after hatch, the tissues were collected from the respiratory and gastrointestinal tracts, and preserved in optimum cutting temperature (OCT) compound at -80°C.

The tissues preserved in OCT were sectioned (thickness of 5µm) and indirect immunofluorescent assay was used to quantify macrophage numbers in respiratory and gastrointestinal tissue sections. For macrophage staining, 5% goat serum in TBS buffer (Trizma base: 2.42g, NaCl: 8g in 1L of distilled water, pH 7.6) was used for the purpose of blocking and incubated at room temperature for 30 mins in a humidified chamber. Unlabeled mouse monoclonal antibody specific for chicken macrophages, KUL01 (Southern Biotech, Birmingham, Alabama, USA) was used in 1:200 dilution in blocking buffer and incubated for 30 mins at the room temperature in a humidified chamber. Then DyLight® 550 conjugated goat anti-mouse IgG (H+L) (Bethyl Laboratories Inc., Montgomery, TX, USA) was used in 1:500 dilution in blocking buffer as the secondary antibody and

incubated for 1 hour at the room temperature in a humidified chamber. Finally, the slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA), cover slipped, sealed with lacquer and fluorescent signals were imaged using an epifluorescence microscope and quantified using Image J software (National Institute of Health, Bethesda, Maryland, USA).

RESULTS

We found that both nucleic acids are capable of eliciting strong innate immune responses characterized by macrophage increase in respiratory and gastrointestinal systems potentially implicating macrophages in the protective host responses against microbial infections in chickens.

REFERENCES

1. Gomis, S., L. Babiuk, B. Allan, P. Willson, E. Waters, N. Ambrose, R. Hecker, and A. Potter. 2004. Protection of neonatal chicks against a lethal challenge of Escherichia coli using DNA containing cytosine-phosphodiester-guanine motifs. Avian diseases 48:813-822.

2. Gordon, S. 2002. Pattern recognition receptors: doubling up for the innate immune response. Cell 111:927-930.

3. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303:1526-1529.

4. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408:740-745.

5. Hofmann, P., H. Sprenger, A. Kaufmann, A. Bender, C. Hasse, M. Nain, and D. Gemsa. 1997. Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. Journal of leukocyte biology 61:408-414.

6. Kameka, A. M., S. Haddadi, D. S. Kim, S. C. Cork, and M. F. Abdul-Careem. 2014. Induction of innate immune response following infectious bronchitis corona virus infection in the respiratory tract of chickens. Virology 450-451:114-121.

7. Kim, H. M., Y. W. Lee, K. J. Lee, H. S. Kim, S. W. Cho, N. van Rooijen, Y. Guan, and S. H. Seo. 2008. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. Journal of virology 82:4265-4274.

8. Krieg, A. M. 2004. Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. Current oncology reports 6:88-95.

9. Kumar, H., T. Kawai, and S. Akira. 2009. Toll-like receptors and innate immunity. Biochemical and biophysical research communications 388:621-625.

10. Medzhitov, R. 2007. TLR-mediated innate immune recognition. Seminars in immunology 19:1-2.

11. Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell 91:295-298.

12. Negash, T., S. O. al-Garib, and E. Gruys. 2004. Comparison of in ovo and post-hatch vaccination with particular reference to infectious bursal disease. A review. The Veterinary quarterly 26:76-87.

13. Seo, S. H., R. Webby, and R. G. Webster. 2004. No apoptotic deaths and different levels of inductions of inflammatory cytokines in alveolar macrophages infected with influenza viruses. Virology 329:270-279.

14. Sharma, J. M., and B. R. Burmester. 1982. Resistance of Marek's Disease at Hatching in Chickens Vaccinated as Embryos with the Turkey Herpesvirus. Avian diseases Vol. 26:134-139.

15. Taghavi, A., B. Allan, G. Mutwiri, A. Van Kessel, P. Willson, L. Babiuk, A. Potter, and S. Gomis. 2008. Protection of neonatal broiler chicks against Salmonella Typhimurium septicemia by DNA containing CpG motifs. Avian diseases 52:398-406.

16. Tarpey, I., and M. B. Huggins. 2007. Onset of immunity following in ovo delivery of avian metapneumovirus vaccines. Veterinary microbiology 124:134-139.

17. Thapa, S., M. S. Cader, K. Murugananthan, E. Nagy, S. Sharif, M. Czub, and M. F. Abdul-Careem. 2015. In ovo delivery of CpG DNA reduces avian infectious laryngotracheitis virus induced mortality and morbidity. Viruses 7:1832-1852.

18. Thapa, S., E. Nagy, and M. F. Abdul-Careem. 2015. In ovo delivery of Toll-like receptor 2 ligand, lipoteichoic acid induces pro-inflammatory mediators reducing post-hatch infectious laryngotracheitis virus infection. Veterinary immunology and immunopathology 164:170-178.

19. Wakenell, P. S., T. Bryan, J. Schaeffer, A. Avakian, C. Williams, and C. Whitfill. 2002. Effect of in ovo vaccine delivery route on herpesvirus of turkeys/SB-1 efficacy and viremia. Avian diseases 46:274-280.

(This article will be submitted as a full-length manuscript to a peer-reviewed journal.)

MODIFIED LIVE INFECTIOUS BURSAL DISEASE VIRUS (IBDV) VACCINE RATHER THAN HERPESVIRUS TURKEY (HVT)-IBDV VECTORED VACCINE DELAYS VARIANT IBDV PATHOGENESIS IN NEONATAL BROILERS

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ABSTRACT

Infection of neonatal chicks with infectious bursal disease virus (IBDV) results in long-lasting immunosuppression and profound economic losses. This study focusing on young age (day-6) infection with variant IBDV (vIBDV) have evaluated the protective efficacy of modified live (MLV) IBDV and herpesvirus turkey (HVT)-IBDV vaccines. We examined IBDV seroconversion, BBW ratio, and bursal histopathology on days 13 and 29 post-challenge. Viral load and T-cell response were assessed using qRT-PCR and flow cytometry, respectively. vIBDV-SK09 challenge caused severe bursal atrophy and lower BBW in HVT-IBDV but not in MLV vaccinated chicks at day 13 post-challenge. Viral load peaked on days 3 and 29 post-challenge in HVT-IBDV and MLV, respectively. Interestingly, our data revealed a previously unrecognized phenomenon that HVT-IBDV but not MLV vaccine decreases T-cell count and suppresses $CD8^+$ T-cell activation in chicks during the critical first week of age. Overall, our data has important implications for vaccine design against IBDV.

INTRODUCTION

Infectious bursal disease (IBD) is a leading cause of immunosuppression in poultry (1-3). IBD in young chicks (less than three weeks of age) causes long-lasting immunosuppression, resulting in tremendous economic losses due to vaccine failures and increased susceptibility to opportunistic pathogens (4). The emergence of varIBDV (5) and very virulent IBDV (vvIBDV) strains (6) which escape MtAb necessitated changes in vaccine regimens. Therefore, in addition to breeder hyper-immunization, broiler vaccination is also being practiced to improve the immunity in chickens against IBDV infection (7, 8). Modified live vaccines (MdLVs) have been introduced and based on intensity of virulence, colloquially classified as mild, intermediate, intermediate plus and hot IBD vaccines. The less attenuated (intermediate and hot) MdLVs induce better protection, but there is a risk that vaccine virus itself can cause bursal damage (9, 10). However, MdLV vaccination by subcutaneous route has been shown to be safe without causing bursal damage (11). The recombinant vectored vaccines are a remarkable accomplishment in vaccine production that combined safety and efficacy in presence of MtAb. Herpes virus of turkey (HVT) has been widely used in conventional vaccination against Marek's disease (12), but also as a recombinant vector for development of a vaccine against IBD (13). Since then, several HVT-IBDV-VP2 vectored vaccines (hereafter referred as HVT-IBDV) have been developed for *in-ovo* or subcutaneous vaccination (14-17). Recent studies in layer (18) and broiler (19) chickens comparing HVT-IBDV and MdLV vaccines suggested that HVT-IBDV is superior to the MdLV vaccine. It is noteworthy to mention that

most of the studies till date examined the protective efficacy of HVT-IBDV and MdLV vaccines (20-22) by challenging birds with pathogenic IBDV at day 18 and 28 (23) or later (24) after the immunization. Efficacy of these vaccines against varIBDV infections occurring in neonatal chicks remains elusive.

MATERIALS AND METHODS

Experimental chickens. Broiler eggs were obtained from a local hatchery (Prairie Pride Chick Sales Ltd., Saskatchewan, Canada), where broiler-breeders undergo routine IBDV (classic strains) hyper-immunization (25). Birds were maintained following the standard procedure as described earlier (25). This study was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Vaccines. Univax-BD, a MdLV purchased from Merck Animal Health, Intervet Inc., Kirkland, QC, and Vaxxitek® (Merial Canada Inc, Baie-D'Urfe, QC), a recombinant HVT-IBD vectored vaccine, carrying the VP2 gene of the classical Faragher 52/70 IBDV strain (26), were used in this study.

Challenge virus. A Canadian field isolate, varIBDV-SK09 (accession number KY352350) was used as the challenge virus (25). The varIBDV-SK09 was passaged for three days in 17-day-old specific pathogen free (SPF) leghorns after an oral infection. The BF were pooled and titrated homogenates were used for the challenge. The embryo infective dose $(EID)_{50}$ was determined using the Reed and Munch method (27).

Real-time reverse transcription PCR (RT-qPCR) and sequence analysis for the quantification of IBDV. The VP2 region of IBDV was PCR amplified (817 bp) and the purified PCR products were sequenced. Based on the predicted amino acids for the VP2 of IBDV, the challenged virus (varIBDV-SK09) was differentiated from the classical vaccine strain.

Flow cytometric analysis. Spleens were excised, and single-cell suspensions were prepared separately, and lymphocytes were separated using Histopaque-1077. Cell preparation and antibody staining for flow cytometry were done, as previously described, with some modifications (28). Flow cytometry data were acquired by EpicsXL (Beckman Coulter) and FACSCaliber (BD Bioscience), and data analyzed with FlowJo software (TreeStar).

Statistical analysis. The BBW, histopathological score, and antibody titer against IBDV were analyzed using Wilcoxon Rank Sum Test (to compare two groups) or Kruskal–Wallis One-way ANOVA (to compare more than two groups). Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) and Statistix7 (Analytical Software, Tallahassee, FL) was used for all the analysis with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Interestingly, histopathological scores and BBW data at 19 days of age revealed that challenge virus (varIBDV-SK09) was able to inflict bursal damage and lymphoid depletion in the HVT-IBDV but not in MdLV vaccinated group. This data suggested that the MdLV vaccine has probably delayed viral pathogenesis. A previous study reported that mild IBDV strain could interfere with a pathogenic IBDV strain infection, and suggested that such interference phenomenon could be either due to competition for host receptor sites or interference by cytokine(s) production (29). Alternatively, MdLV vaccine-induced innate immune response, and T-cell responses could also play a role in restricting the challenge virus from damaging the bursa (30). Our flow cytometric analysis at day eight post-MdLV vaccination revealed an increase in T lymphocytes ($CD4^+$ and $CD8^+$) and $CD8^+$ T-cell activation as evidenced by CD44 upregulation. Previous studies also reported peak T-cell responses against IBDV by seven days post-infection (30, 31). Thus, the delayed varIBDV pathogenesis seen in MdLV group could be the result of viral competition and/or induction of early immune mechanisms. Whatever may be the case, such interference phenomenon may have implications for vaccine-mediated prevention of early age varIBDV infection in broilers.

We carried out RT-qPCR assays to detect viral load kinetics in bursal tissue at nine (three days post infection, dpi), 20 (14 dpi) and 35 (29 dpi) days of age. We could not detect vaccine virus by RT-qPCR either in HVT-IBDV alone or MdLV alone groups throughout our experiment. Previous studies also reported inability to detect the vaccine virus in bursae, which could be due to localization of IBDV vaccine virus in blood or other tissues not investigated here (11). After the challenge, a RT-qPCR analysis revealed low viral load at days nine and 20 of age in the MdLV + varIBDV-SK09 challenged group. However, the MdLV + varIBDV-SK09 challenged group showed an increase in viral load later at 35 days of age. This delayed viral replication is in agreement with our histopathological scores that also showed bursal damage in the MdLV + varIBDV-SK09 challenged group at the later time. Interestingly, three days post-infection with varIBDV-SK09 (nine days of age), the RT-qPCR assays revealed very high viral load in bursal tissues of the HVT-IBDV vaccinated group, which was significantly higher than the unvaccinated + varIBDV-SK09 challenged or MdLV

+ varIBDV-SK09 challenged groups. It was surprising to find that three days after the varIBDV-SK09 challenge, birds which were previously immunized with the protective HVT-IBDV vaccine revealed a significantly higher viral load compared to the unprotected birds (unimmunized control).

Surprisingly, flow cytometric analysis (at day eight of age) of splenic lymphocytes revealed that HVT-IBDV vaccination induced a significant decrease in total $CD4^+$ and $CD8^+$ T-cell numbers, and interestingly also down-regulated CD44 expression (adhesion molecule and activation marker) on cytotoxic $CD8^+$ T-cells. A previous study reported that the HVT vaccine virus had the potential to immunosuppress broiler chickens during the initial three to seven days of age by depleting leucocytes and lymphocytes count in vaccinated birds without affecting humoral response (32). Thus, present data suggest that HVT-IBDV vaccine caused immunosuppression in vaccinated chicks.

It is noteworthy to mention that T-cells are important in limiting IBD disease severity (30), and T-cell suppression leads to an increase in IBDV replication and severe bursal damage (33). Thus, enhanced varIBDV replication in the HVT-IBDV vaccinated group could be due to the HVT induced suppression of leukocytes and lymphocytes (32), leading to decreased immune pressure against varIBDV infection, thereby facilitating rapid early viral growth in the host, consequently early bursal damage. These data suggest that HVT-IBDV vaccine-induced immunosuppression demands strict biosecurity during the first week after the immunization.

In conclusion, the MdLV but not the HVT-IBDV vaccine delayed varIBDV-SK09 pathogenesis, but neither of the vaccines provided complete protection. Our early-age challenge model of a varIBDV infection revealed a previously unrecognized phenomenon that the immunization of broilers with HVT-IBDV induces immunosuppression that may increase susceptibility to varIBDV infection. Overall, our data has important implications for vaccine design against IBDV and optimizing vaccination program in broilers.

ACKNOWLEDGEMENTS

The authors are grateful to animal health technicians at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan. Financial support was provided by grants from Chicken Farmers of Saskatchewan (Saskatchewan Chicken Industry Development Fund, (408996), Saskatchewan Agriculture Development Fund (20100026), Natural Sciences and Engineering Research Council of Canada (CRDPJ 380771-09), Alberta Livestock and Meat Agency (2011F016R) and Alberta Chicken Producers (2011F016R)

REFERENCES

1. Kurukulsuriya S, Ahmed KA, Ojkic D, Gunawardana T, Gupta A, Goonewardene K, et al. Circulating strains of variant infectious bursal disease virus may pose a challenge for antibiotic-free chicken farming in Canada. Res Vet Sci. 2016;108:54-9.

2. Balamurugan V, Kataria JM. The hydropericardium syndrome in poultry--a current scenario. Veterinary Research Communications. 2004;28:127-48.

3. Jackwood DJ. Multivalent virus-like-particle vaccine protects against classic and variant infectious bursal disease viruses. Avian Dis. 2013;57:41-50.

4. Sharma JM, Kim I-J, Rautenschlein S, Yeh H-Y. Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. Developmental & Comparative Immunology. 2000;24:223-35.

5. Snyder DB, Vakharia VN, Savage PK. Naturally occurring-neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. Arch Virol. 1992;127:89-101.

6. Berg TP, Meulemans G. Acute infectious bursal disease in poultry: protection afforded by maternally derived antibodies and interference with live vaccination. Avian Pathol. 1991;20:409-21.

7. Block H, Meyer-Block K, Rebeski DE, Scharr H, De Wit S, Rohn K, et al. A field study on the significance of vaccination against infectious bursal disease virus (IBDV) at the optimal time point in broiler flocks with maternally derived IBDV antibodies. Avian Pathology. 2007;36:401-9.

8. Müller H, Mundt E, Eterradossi N, Islam MR. Current status of vaccines against infectious bursal disease. Avian Pathology. 2012;41:133-9.

9. McCarty J, Brown T, Giambrone J. Delay of infectious bursal disease virus infection by in ovo vaccination of antibody-positive chicken eggs. The Journal of Applied Poultry Research. 2005;14:136-40.

10. Rautenschlein S, Kraemer C, Vanmarcke J, Montiel E. Protective efficacy of intermediate and intermediate plus infectious bursal disease virus (IBDV) vaccines against very virulent IBDV in commercial broilers. Avian diseases. 2005;49:231-7.

11. Knoblich HV, Sommer SE, Jackwood DJ. Antibody titers to infectious bursal disease virus in broiler chicks after vaccination at one day of age with infectious bursal disease virus and Marek's disease virus. Avian Dis. 2000;44:874-84.

12. Zanella A, Granelli G. Marek's disease control: Comparative efficacy of cell-associated and

cell-free lyophilized HVT vaccine. Avian pathology. 1974;3:45-50.

13. Darteil R, Bublot M, Laplace E, Bouquet J-F, Audonnet J-C, Rivière M. Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. Virology. 1995;211:481-90.

14. Le Gros F, Dancer A, Giacomini C, Pizzoni L, Bublot M, Graziani M, et al. Field efficacy trial of a novel HVT-IBD vector vaccine for 1-day-old broilers. Vaccine. 2009;27:592-6.

15. Bublot M, Pritchard N, Le Gros F-X, Goutebroze S. Use of a vectored vaccine against infectious bursal disease of chickens in the face of high-titred maternally derived antibody. Journal of comparative pathology. 2007;137:S81-S4.

16. Tsukamoto K, Saito S, Saeki S, Sato T, Tanimura N, Isobe T, et al. Complete, long-lasting protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. Journal of virology. 2002;76:5637-45.

17. Zhou X, Wang D, Xiong J, Zhang P, Li Y, She R. Protection of chickens, with or without maternal antibodies, against IBDV infection by a recombinant IBDV-VP2 protein. Vaccine. 2010;28:3990-6.

18. Prandini F, Simon B, Jung A, Poppel M, Lemiere S, Rautenschlein S. Comparison of infectious bursal disease live vaccines and a HVT-IBD vector vaccine and their effects on the immune system of commercial layer pullets. Avian Pathol. 2016;45:114-25.

19. Roh JH, Kang M, Wei B, Yoon RH, Seo HS, Bahng JY, et al. Efficacy of HVT-IBD vector vaccine compared to attenuated live vaccine using in-ovo vaccination against a Korean very virulent IBDV in commercial broiler chickens. Poult Sci. 2016;95:1020-4.

20. Rautenschlein S, Yeh HY, Sharma JM. Comparative immunopathogenesis of mild, intermediate, and virulent strains of classic infectious bursal disease virus. Avian Dis. 2003;47:66-78.

21. Rautenschlein S, Kraemer C, Vanmarcke J, Montiel E. Protective efficacy of intermediate and intermediate plus infectious bursal disease virus (IBDV) vaccines against very virulent IBDV in commercial broilers. Avian Dis. 2005;49:231-7.

22. Zorman Rojs O, Krapez U, Slavec B, Jursic-Cizerl R, Poljanec T. Field efficacy of different vaccines against infectious bursal disease in broiler flocks. Acta Vet Hung. 2011;59:385-98.

23. Perozo F, Villegas AP, Fernandez R, Cruz J, Pritchard N. Efficacy of single dose recombinant herpesvirus of turkey infectious bursal disease virus (IBDV) vaccination against a variant IBDV strain. Avian Dis. 2009;53:624-8.

24. Zhou X, Wang D, Xiong J, Zhang P, Li Y, She R. Protection of chickens, with or without maternal antibodies, against IBDV infection by a recombinant IBDV-VP2 protein. Vaccine. 2010;28:3990-6.

25. Kurukulasuriya S, Ahmed KA, Ojkic D, Gunawardana T, Gupta A, Goonewardene K, et al. Circulating strains of variant infectious bursal disease virus may pose a challenge for antibiotic-free chicken farming in Canada. Research in Veterinary Science. 2016;108:54-9.

26. Bublot M, Pritchard N, Le Gros FX, Goutebroze S. Use of a Vectored Vaccine against Infectious Bursal Disease of Chickens in the Face of High-Titred Maternally Derived Antibody. Journal of Comparative Pathology. 2007;137, Supplement 1:S81-S4.

27. Jackwood DJ, Sommer-Wagner SE, Stoute ST, Woolcock PR, Crossley BM, Hietala SK, et al. Characteristics of a very virulent infectious bursal disease virus from California. Avian Diseases. 2009;53:592-600.

28. Ahmed KA, Wang L, Griebel P, Mousseau DD, Xiang J. Differential expression of mannose-6-phosphate receptor regulates T cell contraction. J Leukoc Biol. 2015;98:313-8.

29. Ashraf S, Abdel-Alim G, Al-Natour MQ, Saif YM. Interference between mild and pathogenic strains of infectious bursal disease virus in chickens. Avian Dis. 2005;49:99-103.

30. Kim IJ, You SK, Kim H, Yeh HY, Sharma JM. Characteristics of bursal T lymphocytes induced by infectious bursal disease virus. J Virol. 2000;74:8884-92.

31. Tanimura N, Sharma JM. Appearance of T cells in the bursa of Fabricius and cecal tonsils during the acute phase of infectious bursal disease virus infection in chickens. Avian Dis. 1997;41:638-45.

32. Islam AF, Wong CW, Walkden-Brown SW, Colditz IG, Arzey KE, Groves PJ. Immunosuppressive effects of Marek's disease virus (MDV) and herpesvirus of turkeys (HVT) in broiler chickens and the protective effect of HVT vaccination against MDV challenge. Avian Pathol. 2002;31:449-61.

33. Poonia B, Charan S. T-Cell suppression by cyclosporin-A enhances infectious bursal disease virus infection in experimentally infected chickens. Avian Pathol. 2001;30:311-9.

(This paper has been accepted for the publication in *Vaccine*.)

EVALUATION OF REAL-TIME PCR REAGENTS FOR THE IDENTIFICATION OF INFLUENZA VIRUS RNA

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SUMMARY

IDEXX has developed real-time PCR reagents for the identification of influenza A viral RNA. The RealPCR Influenza A RNA Mix includes primers and probe for an RNA internal positive control (RNA-IPC) to monitor for proper nucleic acid extraction and inhibitors that may be present in the reaction. To date, 217 samples consisting of 18 different hemagglutinin/neuraminidase subtypes sourced from three different host species (swine, avian and canine) have been tested. Results from internal studies suggest the identification of influenza A viral RNA to be highly sensitive and specific when compared to a commercial reference test. The RealPCR Influenza A RNA Mix has been designed to work with the IDEXX RealPCR platform reagents, thus utilizing the same RT-qPCR protocol, a common positive control, and the same RNA master mix used by other RealPCR RNA reagents.

INTRODUCTION

The influenza A virus has proven to be a highly successful pathogen, able to infect a wide range of hosts from swine, avian and canines as well as humans. Rapid detection is usually in the form of virus isolation/detection or real-time PCR. While real-time PCR has offered significant advantages over end-point PCR, commercial real-time PCR assays still use a set of reagents, or "kit", designed for testing a precise number of samples for a specific target(s). This approach often requires a separate testing protocol for each target, increasing time to results and hands-on time for laboratories.

IDEXX RealPCR reagents are all designed to work together. As such, the RealPCR DNA or RNA master mixes may be used with any of the respective DNA or RNA RealPCR target mixes. The single pooled positive control works as a PC for any RealPCR assay. To increase testing efficiency, all RealPCR reagent sets are designed to utilize a single cycling protocol allowing DNA and RNA assays to be run side-by-side. The RealPCR Influenza A RNA reagents described here can be used with any other RealPCR reagents, utilizing the same RealPCR positive control and cycling protocol.

Results demonstrate the performance of the RealPCR Influenza A RNA reagents to be comparable to the reference test, while offering the advantages of being a part of the IDEXX RealPCR platform.

MATERIALS AND METHODS

Sample RNA extraction. RNA was extracted from avian tracheal swabs, canine oral swabs, swine lung lavages or swine oral fluids, using a commercially available magnetic bead protocol. Swabs were eluted individually in 300 µL of PBS. Oral fluids were first clarified via lysis followed by a high-speed centrifugation. Extraction lysis solution was spiked with the RealPCR IPC prior to addition of sample. The RealPCR IPC contains the RNA-IPC target for the RealPCR Influenza A RNA Mix.

Multiplex real-time PCR design. Primers and probes were designed to amplify and identify the presence of a conserved region of the influenza A genome using sequences obtained from positive field samples as well as sequences from the GISAID EpiFlu™ database (http://platform.gisaid.org/epi3/frontend#9c286). The reagents were designed to conform to the RealPCR™ standard cycling protocol. The RNA IPC has been shown by BLAST analysis (1) to have no homology to any sequences in the NCBI database (2).

Real-time PCR standard curves and conditions. Quantified synthetic DNA representing the influenza target sequence was diluted in 10-fold increments to obtain one copy per 5 µL. Amplification reactions were performed in a total volume of 25 µL, with all samples in triplicate. Reactions were incubated at 50°C for 15 minutes, 95°C for 1 minute, followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds with fluorescent signals taken at the end of each extension step in the FAM and HEX channels. Crossing threshold points (Ct) were calculated automatically by the instrument software. Efficiency was calculated using $10^{(-1/\text{slope})} - 1$.

RESULTS

Using a seven log range of 10-fold dilutions, the efficiency was calculated at 101.1% (Figure 1). Although not used in the efficiency calculation, all reactions containing 10 copies of target were detected, while two of three reactions containing one copy of target were detected (data not shown).

A total of 217 samples were extracted for total nucleic acid and tested using the RealPCR Influenza A RNA reagents and a reference influenza A real-time PCR screening test. As shown in Table 1, the RealPCR Influenza A RNA reagents compared nearly 100% with the reference test. One sample that was negative on the RealPCR Influenza A RNA reagents returned a Ct-value of 35.2 using the reference test, suggesting the sample was near the limit of detection for both sets of reagents.

CONCLUSION

Real-time PCR is a powerful tool for the fast detection of nucleic acids. To this end, IDEXX has developed the RealPCR Influenza A RNA reagents to be a part of the RealPCR modular platform, allowing the use of shared reagents and cycling protocols. Testing to date shows the reagents perform with high efficiency over a wide range of target nucleic acid concentrations. Additionally, we have found the reagents were able to detect influenza A viral RNA purified from a wide variety sample types. Finally, our testing showed the detection of influenza A viral RNA using the RealPCR reagents compared favorably to results obtained using a reference influenza A screening test.

REFERENCES

1. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. Basic local alignment search tool. J. Mol. Biol 217:403-410. 1990 215:403-410.

2. NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. Nucleic Acids Research. 2016;44(Database issue):D7-D19. doi:10.1093/nar/gkv1290.

Figure 1. Standard curves for a 10-fold dilutions series of target DNA using RealPCR Influenza A RNA reagents. Efficiency calculated at 101.1% and R^2 value of 0.999 over a seven-log range of samples ending with 100 copies of target per reaction. All reactions containing 10 copies of target were detected and 2/3 reactions containing 1 copy of target were detected (data not shown).

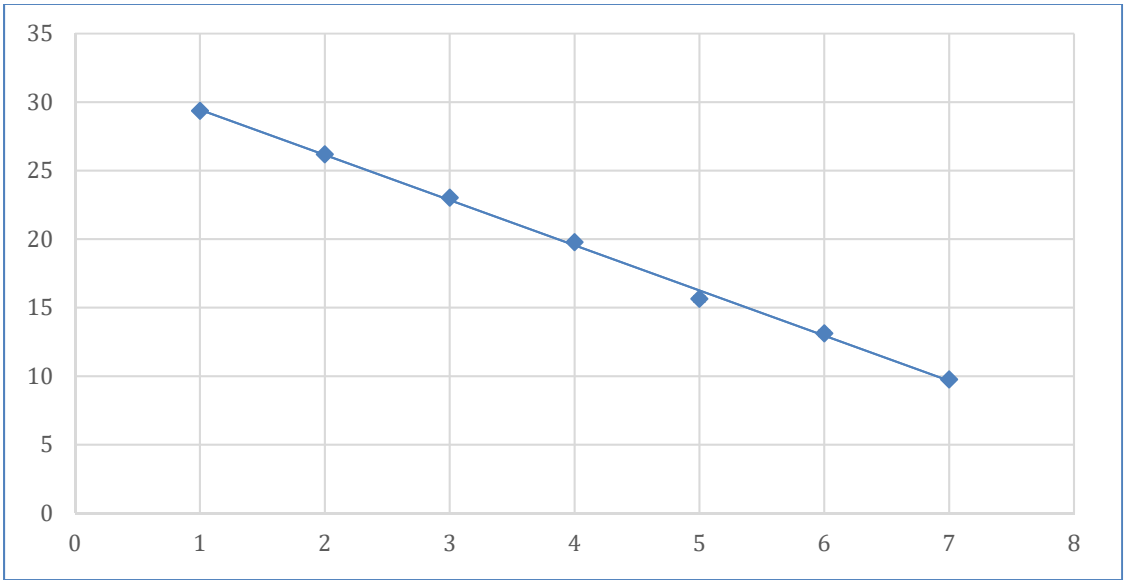

Table 1. Results comparison between RealPCR Influenza A RNA reagents and a reference influenza A real-time PCR screening test. *-Ct of 35.2 using the reference test.

		Reference Method		
		Pos	Neg	Totals
IDEXX RealPCR	Pos	106	0	106
	Neg	1*	110	111
	Totals	107	110	217

STUDY OF PATHOGENICITY OF EMERGING AVIAN REOVIRUS VARIANTS ISOLATED FROM BROILER CHICKENS

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ABSTRACT

This study examined the pathogenicity of emerging avian reoviruses (ARVs) isolated from clinical cases of tendinitis in broiler chickens from Saskatchewan, Canada. Week old SPF chickens were divided into five groups. Each of the four groups were infected with 10^5 $TCID_{50}$ of a different strain of ARV variant via the foot pad route at the right leg. The fifth group was mock infected with saline. The development of clinical disease and the pattern of disease progression was observed every day for 45 days. All the four ARV variants were capable of causing clinical disease. The prominent clinical signs observed were swelling of the foot pad and the tarsometatarsal joint, lameness and ruffled feathers. The tendon sheaths of infected birds were expanded to varying degree by infiltrating mononuclear cells, mainly lymphocytes, plasma cells and moderate number of heterophils. Increased fibroplasia and collagen deposition were observed at the later stages of infection. In addition, there was an increased level of gamma-delta T cells, CD8 T cells and MHC-II expressing macrophages at day nine post infection with a high degree of apoptosis of non-immune cells. At 45 days post infection, infected birds experienced retarded growth with considerable reduction in live weight.

INTRODUCTION

ARVs are grouped under the *orthoreovirus* genus in the family *Reoviridae*. ARVs are non-enveloped viruses with icosahedral double capsid containing ten segments of double stranded RNA (dsRNA) genome. The genomic segments are divided into three size classes (i.e. Large [L], Medium [M] and Small [S]) based on their electrophoretic mobility on a polyacrylamide gel (6). The ARV genome encodes four non-structural proteins (μNS, μNS, P10 and P17) and eight structural proteins (λA, λB, λC, μA, μBC, σA, σB and σC (1).

Arthrogenic ARVs cause tenosynovitis/arthritis syndrome which is characterized by unilateral or bilateral swelling of the hock joint resulting in lameness. The disease mainly affects younger birds and is associated with poor growth and sometimes death due to the inability of affected birds to move towards feed and water. Consequently, the disease causes significant economic losses to the broiler poultry industry. In Saskatchewan, Canada the there has been an increase trend in the diagnosis of the disease since 2012 despite regular vaccinations using commercially available ARV vaccines.

Recently, we isolated and characterized emerging ARVs in Saskatchewan, Canada from samples collected in 2013, 2014 and 2015. Our isolates were clustered into four distinct genotyping groups and found to be different from the vaccine strains. In addition, none of the isolates were neutralized by antibodies produced against the commercially available vaccines. Therefore, this study was conducted with the objective of investigating the pathogenicity of the ARVs isolated from clinical cases of arthritis in broiler chickens in Saskatchewan, Canada.

METHODOLOGY

Viruses. Emerging avian reovirus variants that were isolated from clinical cases of tendinitis in broilers in Saskatchewan were used in this study.

Animal experiment. Day-old broiler chickens were divided in to five groups, 12 birds in each group. The groups were kept in separate isolation units in the WCVM animal care unit and provided with ample food and water. Each of the four group of birds were infected with a different strain of reovirus via the right footpad using 25 gauge needle, the fifth group was injected with saline and kept as a negative control. The birds were monitored three times a day and any abnormality observed was recorded. Three animals from each group were euthanized at 3, 9, 17 and 45 days post virus infection and samples were collected for laboratory analysis. The animal experiment was approved by the University of Saskatchewan's Animal Research Ethics Board.

Gross and histopathology. The birds were examined for any gross lesions before and after euthanization. Sections of tendon tissues from the right leg were fixed in 10% neutral buffered formalin. The fixed tissues were embedded in paraffin, sectioned at 5μm thickness, stained with hematoxylin and eosin, and examined under a light microscope.

Immunogold staining and electron microscopy. Tendon tissue samples were ultrathinsectioned and blocked with 1% (w/v) Y-globulin free serum albumin three times, 10 min each. Immunogold staining was performed as described previously (2) using anti-avian reovirus primary antibody and a gold conjugate secondary antibody. Later, the samples incubated with 20 µL drops of 2% aqueous uranyl acetate with a drop of triton X-100 for 20 min followed by 4 x 4 min washes in distilled water. The excess water was blotted with Kimwipes after the final wash and the grids were incubated with Renold's lead citrate for 10 min followed by 5 x 4 min washes with water. Finally, the sections were blotted with Kimwipes, put on grid grippers and observed under electron microscopy.

Flow cytometry. Tendon samples were incubated with collagenase (Sigma Aldrich) at a concentration of 2mg/ml in 1XPBS for 30 min at 37°c. Single cell suspensions were collected and washed twice with 1X PBS containing 0.1% sodium azide and 1% bovine calf serum. Subsequently, the cells were incubated with primary antibodies specific for chicken immune cells for 30 min on ice, followed by incubation with fluorescent conjugated secondary antibodies. Finally, the cells were washed twice and re-suspended in 1XPBS-azide and analyzed using a Flow cytometer (Beckman coulter). FlowJo software was used for data analysis.

Apoptosis assay. Single cell suspensions of virus infected tendon tissue were incubated with fluorescent dye conjugated primary antibodies that are specific for chicken T-cells, B-cells or macrophages. The cells were washed two times in PBS and re-suspended in 100uL of 1X binding buffer (Kingfisher Biotech, Inc) and 10uL of a working solution of chicken Annexin V fluorescein (Kingfisher Biotech, Inc), After 15 min of incubation at room temperature in the dark, 400uL of 1X binding buffer was added to each tube and immediately analyzed by Flow cytometry.

RESULTS AND DISCUSSION

All the ARV strains from each cluster group were capable of causing clinical disease. The disease, in all cases, was initially characterized by swelling of the foot pad which later extended to the tarsometatarsal joint. After 48 hours post infection, all infected birds were inactive with ruffled feathers and were observed in a sitting position. When stimulated to react, they demonstrated lameness and some were reluctant to move.

At necropsy, inflammation of the foot pad extending up to the hock joint was observed including the synovial membranes and the surrounding tissue. No significant lesions were observed in internal organs. Based on gross lesions, there was no significant difference in the severity of the disease induced by the different ARV strains.

On histopathology, as compared to tendons from healthy birds, the tendon sheaths of infected birds were thickened with lymphocytic-plasmacytic infiltration. At later stages post infection, increased fibroplasia and collagen deposition were observed.

Similar gross and microscopic abnormalities were observed by other pathogenic strains of ARV (3, 7). As compared to uninfected negative control birds, the average weight of the birds in infected groups reduced to varying degrees ranging from 10.57% to 29.58% at 45 days post infection.

In a previous study, a significant reduction in body weight was reported after 12 and 16 weeks of age following experimental inoculation of turkeys with turkey reovirus (4). This was attributed to pain and discomfort associated with lameness that makes it difficult for the birds to reach food and water.

Virus was detected in the tendon tissues at 3, 9 and 17 days post infection in all infected groups, but not at 45 days by immunogold-staining electron microscopy. Flow cytometry analysis of single cell suspension of tendon tissues revealed a significant increase in the level of gamma delta T cells, CD8 T cells and MHC-II expressing macrophages at 9 days post infection in all infected groups. No significant difference was observed in the level of B-cells between infected and non-infected negative controls. As observed by (5), a significant level of IL-10 and INF-γ was detected in tendon tissues of all infected groups. It is previously reported that a higher level of replication of ARVs in tendon tissues of chickens induces the production of significant levels of IL-10 and INF-γ (5). In addition, an increase in the level of IL-2 in infected tendon tissues indicates an increase in the proliferation of lymphocytes, which is in correlation with the histopathology and flow cytometry results. Infection of tendon tissues with ARV also induced apoptosis of non-immune cells, which might be a mechanism of cell killing employed by ARVs for progeny virus release and virus spread.

In conclusion, all variant strains of ARV isolated from clinical cases in broilers in Saskatchewan are pathogenic and were able to produce clinical disease. Further studies on immuno-pathogenesis and mechanism of apoptosis are essential in the development of effective disease control strategies.

REFERENCES

1. Bodelon, G., Labrada, L., Martinez-Costas, J. and Benavente, J. The avian reovirus genome segment S1 is a functionally tricistronic gene that express one

structural and two non-structural proteins in infected cells. Virology. 290: 181-191. 2001.

2. Deshmukh, D.R., Dutta, S.K. & Pomeroy, B.S. Avian Reoviruses V. Studies of Ultrastructural Morphology by Electron Microscopy. Avian Dis. 15 (3): 588-595. 1971.

3. McNulty, M.S. Reovirus. In Vims infections in birds (J.B. MeFerran & M.S. McNulty, eds). Elsevier Science Publishers BV, Amsterdam, 181-193. 1993.

4. Sharafeldin, T.A., Mor, S.K., Bekele, A.Z., Verma, H., Noll, S.L., Goyal, S.M., Porter, R.E. Experimentally induced lameness in turkeys inoculated with a newly emergent turkey reovirus. Vet Res. 46:11. 2015.

5. Shen, P., Yang, J., Su, B. and Lee, L. Cytokine mRNA expression in chicken experimentally infected with different avian reovirus strains. Taiw. *Vet. J.* 40: 29–36. 2014.

6. Spandidos, D.A. and Graham, A.F. Physical and chemical characterization of an avian reovirus. *J Virol.* 19(3): 968-76. 1976.

7. Van der Heide, L. Tenosynovitis/viral arthritis. A review. Avian Pathol. 6: 271-284. 1977.

HIGH MORTALITY ASSOCIATED WITH *ESCHERICHIA COLI* IN QUAIL: CASE REPORT

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SUMMARY

Twenty eight-weeks-old quail from a farm located at Guanajuato, Mexico showing signs of prostration, ruffled feathers, granulomas, decreased production parameters and increased mortality were submitted to a diagnostic laboratory in Jalisco. Affected birds were undersized for the flock, with cannibalism injuries by other birds. Birds were often dying, inactive, not eating, dehydrated, and not reacting to stimulation.

Samples of lung, liver, spleen and bone marrow were collected for bacterial isolation. Thirty-one *Escherichia coli* strains were isolated from eighteen birds, most of them recovered from spleen samples, followed by liver and lung. All samples of bone marrow were negative for the isolation. The identification was made with biochemical conventional test. Material of the samples were inoculated, first on infusion heart-brain broth and incubated aerobically for 12 hours at 37ºC, later was streaked on MacConkey agar in the same conditions, there was possible obtain colonies characteristically bright pink lactose-fermenters, with a precipitate surrounding colonies, but also was some colonies non-lactose fermenters.

Biochemical features was obtained through use of Kligler´s iron agar, citrate (Simmons), sulfide-indole-motility (SIM) medium, methyl red, Voges-Proskauer, malonate- phenylalanine, gluconate, and urea. Biochemical properties accord with *E. coli.*

We proceeded to make the antimicrobial susceptibility tests in Mueller-Hinton agar (MHA). For the inoculation was prepared the inoculum with suspension equivalent to a 0.5 McFarland standard, the incubation to 37°C for 16 to 20 hours. Antimicrobial agent used for the test: Amikacin (AK), ampicillin (AMP), amoxicillin-clavulanate (AMC), aztreonam (ATM), cefepime (FEP), cefotaxime (CTX), ceftriaxone (CRO), cefoxitin (FOX), ceftazidime (CAZ), ceftiofur (EFT), ciprofloxacin (CIP), doxycycline (D), enrofloxacin (ENR), florfenicol (FLD), imipenem (IPM), lincomycin (L), nalidixic acid (NA), neomycin (N), novobiocin (NB), norfloxacin (NOR), ofloxacin (OFX), oxytetracycline (OXY), tetracycline (TE), trimethoprim-sulfamethoxazole (STX) and sulfachloropyridazine (SCP).

For the interpretation of the results the Clinical and Laboratory Standards Institute, M100-S25 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement January 2015 was considered. Table 1 shows (in percentage) the results of resistance, susceptibility or if it was considered intermediate.

Important levels of antimicrobial resistance were seen among the isolates in this case, especially the antibiotics more used in poultry.

These results overall with the clinical signals suggest that *E. coli* can be a primary pathogen in quails.

As we know the antimicrobial resistance is determined genetically and is usually transferable within a species or between different types of bacteria via mobile genetic elements, this capacity was caused a growing concern over antibiotic resistance, especially in case of multidrug resistance, that case could be noticed in this isolations. Table 2 presents the results for each strain in the antimicrobial susceptibility tests.

Multi-drug resistance is considered more important for the potential of bacterial strains that affect people who acquire bacterial resistance factors in animals, leading to changes in the way antimicrobials are used to treat diseases in poultry. That is part of the motivation to continue research for new alternatives to treat colibacillosis. Serotyping and virulence gene detection will be performed and those results will be presented later.

Table 1. Results for antimicrobial susceptibility testing.

Antimicrobial agent	%	Susceptible	Medium	Resistant
Amikacin	AK 30	93.55	3.23	3.23
Ampicillin	AMP	3.23	0	96.77
Amoxicillin - Clavulanate	AMC	16.23	80.65	3.23
Aztreonam	ATM	100	0	0
Cefepime	FEP	32.26	67.74	0
Cefotaxime	CTX	64.52	25.81	9.68
Ceftriaxone	CRO	100	0	0
Cefoxitin	FOX	29.03	61.29	9.68
Ceftazidime	CAZ	100	0	0
Ceftiofur	EFT	58.06	35.48	6.45
Ciprofloxacin	CIP	51.61	16.13	32.26
Doxycycline	D	0	0	100
Enrofloxacin	ENR	16.16	22.58	61.29
Florfenicol	FLD	6.45	6.45	87.1
Imipenem	IPM	96.77	3.23	0
Lincomycin	L	0	0	100
Nalidixic Acid	NA	0	0	100
Neomycin	N	29.03	32.26	38.71
Novobiocin	NB	0	0	100
Norfloxacin	NOR	58.06	9.68	32.26
Ofloxacin	OFX	54.84	6.45	38.71
Oxytetracycline	OXY	0	0	100
Tetracycline	TE	0	0	100
Trimethoprim- sulfamethoxazole	STX	38.7	0	61.3
Sulphachlorpyridazine	SCP	19	13	68

Table 2. Results for each *Escherichia coli* strain.

#	Sample	AK	AMP	AMC	ATM	FEP	CTX	CRO	FOX	CAZ	EFT	CIP	D	ENR	FLD	IPM	L	NA	N	NB	NOR	OFX	OXY	TE	STX	SCP
1	Spleen	S	S	I	S	I	I	S	R	S	I	R	R	R	R	S	R	R	I	R	R	R	R	R	R	R
2	Spleen	S	R	I	S	S	S	S	I	S	S	R	R	R	I	S	R	R	S	R	R	R	R	R	R	R
3	Spleen	S	R	I	S	S	S	S	I	S	I	R	R	R	I	S	R	R	S	R	R	R	R	R	R	R
4	Spleen	S	R	I	S	S	S	S	I	S	I	R	R	R	R	S	R	R	I	R	R	R	R	R	R	R
5	Spleen	S	R	R	S	S	S	S	I	S	I	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
6	Liver	S	R	I	S	S	S	S	S	S	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
7	Spleen	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	R	R	R	I	S	R	R	R	R
8	Spleen	S	R	S	S	S	S	S	S	S	S	S	R	R	S	S	R	R	S	R	I	S	R	R	R	R
9	Spleen	S	R	S	S	S	S	S	S	S	S	S	R	R	R	S	R	R	S	R	S	S	R	R	R	R
10	Lung	S	R	S	S	S	S	S	S	S	S	S	R	R	R	S	R	R	I	R	S	S	R	R	R	R
11	Spleen	S	R	S	S	S	S	S	S	S	S	I	R	R	R	S	R	R	S	R	S	S	R	R	R	R
12	Liver	S	R	I	S	I	I	S	S	S	I	R	R	R	R	S	R	R	I	R	R	R	R	R	R	R
13	Lung	S	R	I	S	I	I	S	I	S	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
14	Spleen	S	R	I	S	I	I	S	S	S	I	S	R	I	R	S	R	R	S	R	S	S	R	R	S	I
15	Spleen	S	R	I	S	I	I	S	I	S	I	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
16	Liver	S	R	I	S	I	R	S	I	S	I	S	R	S	R	S	R	R	I	R	S	S	R	R	S	I
17	Liver	S	R	I	S	I	R	S	I	S	S	S	R	I	R	S	R	R	S	R	S	S	R	R	S	S
18	Spleen	S	R	I	S	I	I	S	I	S	I	S	R	S	R	S	R	R	I	R	S	S	R	R	S	I
19	Liver	S	R	I	S	I	I	S	I	S	S	S	R	S	R	S	R	R	R	R	S	S	R	R	S	R
20	Lung	S	R	I	S	I	S	S	I	S	S	S	R	I	R	S	R	R	S	R	S	S	R	R	S	S
21	Liver	S	R	I	S	I	S	S	I	S	S	S	R	S	R	S	R	R	S	R	S	S	R	R	S	S
22	Spleen	S	R	I	S	I	R	S	R	S	S	I	R	R	R	I	R	R	R	R	S	I	R	R	R	R
23	Spleen	S	R	I	S	I	S	S	I	S	S	I	R	R	R	S	R	R	R	R	I	I	R	R	R	R
24	Liver	S	R	I	S	I	S	S	I	S	S	I	R	R	R	S	R	R	R	R	S	R	R	R	R	R
25	Spleen	S	R	I	S	I	I	S	R	S	I	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
26	Spleen	S	R	I	S	I	S	S	I	S	I	I	R	R	R	S	R	R	R	R	S	R	R	R	R	R
27	Spleen	R	R	I	S	I	S	S	S	S	S	S	R	I	R	S	R	R	R	R	S	S	R	R	S	I
28	Spleen	I	R	I	S	I	S	S	I	S	R	S	R	I	R	S	R	R	I	R	S	S	R	R	S	R
29	Spleen	S	R	I	S	I	S	S	I	S	S	S	R	R	R	S	R	R	I	R	S	S	R	R	S	S
30	Spleen	S	R	I	S	I	S	S	I	S	S	S	R	I	R	S	R	R	I	R	S	S	R	R	S	S
31	Spleen	S	R	I	S	I	S	S	I	S	S	S	R	I	R	S	R	R	I	R	S	S	R	R	S	S

BLOOD CHEMISTRY REFERENCE INTERVALS FOR BACKYARD HENS

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SUMMARY

Keeping backyard poultry has become increasingly popular in urban and suburban households. Some have dubbed urban chickens as the mascot of the "buy local" and urban sustainability movements (1). Backyard poultry owners often consider their chickens as pets (4), and they may not be amenable to sacrificing one or two chickens for the sake of a diagnosis. Therefore, it is imperative that veterinarians are equipped with ante-mortem diagnostics, such as serum biochemical profiling, to serve the backyard chicken demographic. Veterinarians who are willing to serve this demographic need lab reference intervals that capture the range of clinically healthy backyard hens.

Currently, reference intervals for biochemical parameters in backyard chickens are limited. Published reference values for the *Gallus gallus domesticus* can be found in the *Exotic Animal Formulary* (2) and *Schalm's Veterinary Hematology* (8), but the origin of these intervals could not be traced. Although reference intervals for commercial laying hens and broiler breeders have recently been published (5, 7), nutrition, management, and genetics of these commercial strains are very different from backyard flocks. The purpose of this study was to develop blood chemistry reference intervals for use in backyard flocks.

Between June and August 2016, 133 hens from 34 different flocks in Western Washington were sampled via medial metatarsal venipuncture. Whole heparinized blood was analyzed using a VetScan VS2® with Avian/Reptilian Profile Plus reagent rotors. Packed cell volume was determined via centrifugation of microhematocrit tubes. The following reference ranges were calculated by Reference Value Advisor V2.1 software using the non-parametric method: AST 117.6 - 297.9 U/L; Bile Acids ≤44.6 µmol/L; Creatine Kinase 107.4 - 1780.3 U/L; Uric Acid 0.86 - 8.91 mg/dL; Glucose 174.3 - 239.3 mg/dL; Calcium ≥10.93 mg/dL; Phosphorus 1.60 - 7.23 mg/dL; Potassium 3.17 - 6.10 mmol/L; Sodium 133.3 - 150.8 mmol/L; Total Protein 3.85 - 6.98 g/dL; Albumin 1.50 - 3.30 g/dL; Globulin 1.64 - 4.30 g/dL; PCV 24 - 36 %. Seven out of ten currently published reference intervals for the *Gallus gallus domesticus* were not validated for use in backyard hens, according to the guidelines established by the Clinical and Laboratory Standards Institute (3).

It is important for veterinarians to understand the attitudes and motives of small flock owners and to offer the same caliber of diagnostics and care as they do for traditional pets. Because clinical signs of illness in avian species are often subtle, blood chemistry profiles may be helpful in diagnosing disease (6), especially with 47% of flock owners reporting that they would consider paying for the test if a veterinarian thought it would be beneficial.

REFERENCES

1. Block, B. U.S. city dwellers flock to raising chickens. World Watch Institute. [internet]. [cited 2016 Oct 2]. Available from: http://www.worldwatch.org/node/5900. 2016.

2. Carpenter, J. W. and C. J. Marion. Exotic Animal Formulary. 4th ed. St. Louis, MO: Elsevier, 2013.

3. CLSI. Defining, establishing, and verifying reference intervals in the clinical laboratory; approved guideline. 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute. 2008.

4. Elkhoraibi C., R. A. Blatchford, M.E. Pitesky, and J. A. Mench. Backyard chickens in the United States: A survey of flock owners. Poult. Sci. 93:2920-2931. 2014.

5. Martin, M. P., M. Wineland, and H. J. Barnes. Selected blood chemistry and gas reference ranges for broiler breeders using the i-STAT® handheld clinical analyzer. Avian Dis. 54:1016-1020. 2010.

6. Ritchie, B. W., G. J. Harrison, and L. R. Harrison (eds). Avian Medicine: Principles and Application. Lake Worth, FL: Wingers Publishing. 1994.

7. Schaal, T. P., J. Arango, A. Wolc, et al. Commercial Hy-Line W-36 pullet and laying hen venous blood gas and chemistry profiles utilizing the portable i-STAT®1 analyzer. Poult. Sci. 95:466-471. 2016.

8. Wakenell, P. Normal Avian Hematology: Chicken and Turkey. In: Schalm's Veterinary

Hematology. 6th ed. Weiss, D.J. and K. J. Wardrop (eds). Baltimore, MD: Lippincott Williams & Wilkins. pp 958-967. 2010.

OREGANO ESSENTIAL OIL PRODUCT REDUCES BCO LAMENESS

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ABSTRACT

Lameness can be a significant issue for bird welfare and performance. Bacterial chondronecrosis with osteomyelitis (BCO) is recognised as one of the key causes of lameness and mortality in broilers, and a common problem for poultry operations. *Staphylococcus agnetis* is the predominant species that has been isolated from BCO lesions of lame broilers in experimental models. BCO lameness is thought to primarily occur following a breakdown in gut health and integrity, which allows *S. agnetis* to translocate from the intestine to the proximal femoral and tibial heads. Orego-Stim (Anpario, UK) is an oregano oil-based feed additive that has been shown to promote a favourable gut environment and intestinal health. This study determined the effects of Orego-Stim on lameness in broilers in a *S. agnetis* challenge model. A total of 480 Cobb 500 birds were split into two groups (4 replicates/group). The two groups were; control (C) and Orego-Stim at 500g per tonne of feed. Birds were provided with a nutrient sufficient diet, and water, *ad-libitum*. Birds were raised from days 1-56 on wire flooring. On Days 20 and 21, birds were challenged via drinking water with *S. agnetis* isolate 908 at 10^5 CFU per mL of drinking water. Birds were assessed for lameness twice a day from Day 22. Those birds deemed clinically lame were humanely euthanized, necropsied and cause of lameness determined. Birds that died naturally were also necropsied and assessed. During the 56 day experiment, Orego-Stim reduced the incidence of lameness (Orego-Stim: 57% vs C: 77%; $P<0.001$) and increased final bodyweight (Orego-Stim: 3.86kg vs. 3.52kg; $P<0.05$). This work demonstrated that Orego-Stim reduced broiler lameness in a BCO-induced model, which, based on previous work, may be due to beneficial effects on gut integrity and health.

INTRODUCTION

Lameness is a significant issue for the poultry industry and compromises bird health, welfare and performance. BCO is recognised as one of the key causes of lameness and mortality in broilers and recent reports suggest that more than 1% of all broilers grown to heavier weights are affected during their lives (1). Rapid broiler growth rates can expose the proximal femorae and tibiae to excessive mechanical stresses that create wound sites that become infected by blood-derived opportunistic bacteria (1). In studies where lameness has been induced at high levels, the primary bacterial species isolated from BCO lesions is *Staphylococcus agnetis* (2). Although other bacteria can induce BCO lameness, S. agnetis is an effective model for studying BCO etiology. Compromised intestinal integrity and health is considered to be a factor allowing *S. agnetis* to translocate from the intestine to the femoral and tibial heads.

Orego-Stim is an oregano oil-based additive, which has the monoterpene compounds carvacrol and thymol as the primary constituents. Orego-Stim has been demonstrated to be beneficial for intestinal infections in broilers, reducing intestinal (coccidial) lesions and improving performance (3). Moreover, Orego-Stim has been shown to modify the intestinal microbiota (4) and stimulate enterocyte proliferation (5). Therefore, Orego-Stim helps to promote the integrity and health of the intestine, which may prevent bacterial translocation.

This study investigated the effects of Orego-Stim on lameness in broilers using a *S. agnetis* challenge model.

MATERIALS AND METHODS

Four hundred and eighty Cobb 500 male birds were split between two treatment groups with four replicates of 60 birds per group. All birds were raised on wire flooring (as described previously (1)) and were 'walked' daily by being prompted with a broom. One treatment group (control) received a basal corn-soybean meal-based feed, while the other group received the same basal feed but supplemented with 500 g per tonne of Orego-Stim (Anpario, UK). The basal feed was formulated to meet or exceed the nutritional requirements of the birds. On days 20 and 21, all birds were challenged by administration of *S. agnetis* isolate 908 at 10^5 CFU per ml in their drinking water. From day 22 onwards, all birds received standard drinking water without artificial contamination. Feed and water were provided *ad*

libitum. Birds continued to be 'walked' and were observed for lameness twice per day from day 22. Birds unwilling or unable to walk were deemed clinically lame and humanely euthanized. All birds that died or developed clinical lameness were necropsied and categorised according to the necropsy observations. On day 56, surviving birds were weighed, then euthanized and necropsied to assess femoral or tibial sub-clinical lesion incidences. Cumulative lameness during the 56 day experiment was determined. Environmental temperature, photoperiod (23 hour light: one hour dark) and ventilation were computer controlled during the study.

Treatment affects were evaluated for significant differences ($P<0.05$) according to a logistic regression (binomial distribution) using the GLM procedure implemented in R-3.3.1 (R Foundation for Statistical Computing).

RESULTS

During the 56 day experiment, Orego-Stim reduced the cumulative incidence of broiler lameness compared to the control group by 20 percentage points (57% vs. 77%, respectively ($P<0.001$)). The pattern of cumulative lameness is shown in Figure 1.

For birds that were diagnosed as lame, there were no treatment differences with regards to the bones involved (i.e. femorae or tibiae) or in the severity of the BCO lesions ($P>0.05$).

On day 56, Orego-Stim fed birds had a higher final bodyweight compared to control birds (3.86 kg vs 3.52 kg, respectively ($P<0.05$)).

DISCUSSION

Raising broiler chickens on wire flooring is an established method for inducing BCO lameness, which can be exacerbated by oral challenge with *S. agnetis* (1). The model used in this experiment successfully induced widespread lameness. The addition of Orego-Stim to the feed of *S. agnetis* challenged broilers at 500g per tonne effectively reduced lameness during the study. Compromised intestinal integrity and health represent the obvious means by which orally administered *S. agnetis* may translocate from the gastrointestinal tract to the femoral and tibial heads and thus cause BCO lameness. Therefore, factors that impair the intestinal barrier, such as enteric infections, may exacerbate *S. agnetis* translocation and BCO lameness, while strategies or additives that promote an effective barrier may thus reduce the development and onset of lameness. Orego-Stim has been shown to be effective for mitigating the effects of enteric diseases, such as necrotic enteritis (6) and coccidiosis (3, 7), in broilers. Moreover, oregano oil and its major constituents, carvacrol and thymol, have been shown to be effective antimicrobials (8) and antioxidants (5), with the ability to stimulate enterocyte proliferation (5). Clearly, Orego-Stim has features that would help promote intestinal health and integrity of the gut barrier. Therefore, it can be hypothesised that these benefits of Orego-Stim reduced *S. agnetis* translocation from the intestine and reduced the occurrence of lameness. The effects of Orego-stim via the feed would be expected to be focussed on the intestine and localised tissues. It is, therefore, perhaps not unexpected that Orego-Stim did not influence the affected bones or severity of BCO lesions in birds where the gut barrier had been breached and lameness had occurred.

A number of studies have reported improvements in animal performance when supplemented with Orego-Stim (4, 5), including following challenge (3), and so the improvement in final bodyweight in this study is not unexpected. The potential improvements in intestinal health, which likely contributed to reduced lameness development, would also be of benefit in allowing the birds to utilise dietary nutrients more efficiently for growth.

In conclusion, Orego-Stim reduced broiler lameness and increased final bodyweight in a BCO-induced model, which it can be hypothesised resulted from positive effects on intestinal integrity and health.

REFERENCES

1. Wideman, R. F. Bacterial chondronecrosis with osteomyelitis and lameness in broilers: a review. Poult. Sci. 95:325–344. 2016.

2. Al-Rubaye, A. A. K., Couger, M. B., Koon, J. A. II, Ojha, S., Pummill, J. F., R. F. Wideman Jr and D. D. Rhoads. Genome analysis of *staphylococcus agnetis*, an agent of lameness in broiler chickens. PLoS ONE 10(11): e0143336. doi:10.1371/journal.pone.0143336. 2015.

3. Mohiti-Asli, M., and M. Ghanaatparast-Rashti. Dietary oregano essential oil alleviates experimentally induced coccidiosis in broilers. Prev. Vet. Med. 120:195–202. 2015.

4. Tan, C., J. Ao, S. Jiang, G. Long, J. Peng, H. Sun, and H. Wei. Effects of supplementation of oregano essential oil to sows on oxidative stress, lactation feed intake of sows and piglet performance. Biomed. Res Int. 2015, Article ID 525218, http://dx.doi.org/10.1155/2015/525218.

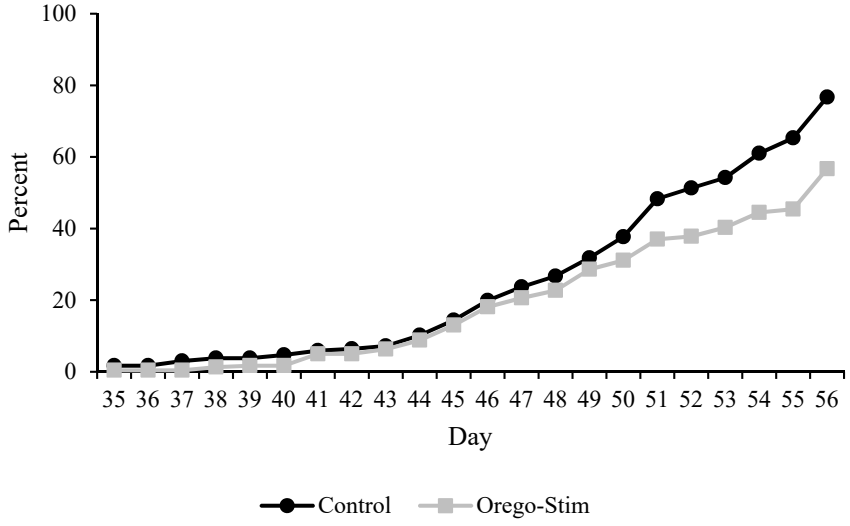
5. Konstanti, A. E., L. J. Broom, I. Giannenas, D. Harrington and A. Owusu-Asiedu. Commercial oregano product increases intestinal cell proliferation, antioxidant capability and performance of broilers. Poult. Sci. 95(E-Suppl. 1): pp. 82. 2016.

6. Saini, R., S. Davis, and W. Dudley-Cash. Oregano essential oil reduces necrotic enteritis in broilers. Abstract. Proc. 52nd Conference on Western Poultry Diseases, Sacramento. CA. pp. 95–97. 2003.

7. Saini, R., S. Davis, and W. Dudley-Cash. Oregano essential oil reduces the expression of coccidiosis in broilers. Abstract. Proc. 52nd Conference on Western Poultry Diseases, Sacramento. CA. pp. 97–98. 2003.

8. Mith, H., Clinquart, A., Daube, G., Delcenserie, V., R. Dure and A. Zhiri. Antimicrobial activities of commercial essential oils and their components against food-borne pathogens and food spoilage bacteria. Food Sci. Nutr. 2:403–416. 2014.

Figure 2. Cumulative percent lameness by treatment group through day 56. Values are means from the four pens per group. Groups are statistically different, $P \leq 0.001$.



IN ARGENTINA, SOME MAREK'S DISEASE VACCINES ARE CONTAMINATED WITH CHICKEN INFECTIOUS ANEMIA VIRUS AS SHOWN BY ANTIBODY SURVEY IN DOMESTIC POULTRY AND FREE LIVING WILD BIRDS

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INTRODUCTION

Chicken infectious anemia (CIA) was first recognized by Yuasa *et al*. in 1979 as a new disease in young chickens caused by a novel virus (CIAV). Since the first description of this disease and subsequent isolation of the virus in cell culture in Japan, CIAV has been isolated in virtually all countries with a poultry industry. CIA is characterized by aplastic anemia and generalized lymphoid atrophy with concomitant immunosuppression and is frequently complicated by secondary viral, bacterial, or fungal infections. CIAV plays a major role in the etiology of a number of multifactorial diseases associated with hemorrhagic syndrome and/or aplastic anemia. In addition to anemia and associated syndromes, subclinical chicken infectious anemia virus infections, without anemia and increased mortality, are frequently observed in commercial flocks (12). In Argentina, CIA was identified in 1991 and the virus was isolated for the first time in 1993 (5). Since then, other isolates have been reported (1, 2) and several others have been characterized (10). In 1997 Buscaglia and Tohya reported a serological survey for antibodies in commercial poultry (4) and Buscaglia showed, at the IXth Argentinean Virology Congress, the presence of CIAV antibodies in backyard hens provided to poor families in a social program call Pro Hueta (3). The results of serological tests on sera obtained from backyard chickens from different locations of the Buenos Aires Province unrelated to the social program were presented in part and combined with the data from sera from the social program that was presented, but not published in detail yet (4). More sera unrelated to the social program were obtained from small flocks of chickens that were distant from commercial poultry operations. Results from samples obtained from wild birds from the province of Buenos Aires will be presented and compare to sera from both types of backyard chickens and commercial poultry from the provinces of Buenos Aires and Entre Rios. In summary the aim of the present study was to determine the presence of antibodies to CIAV in free living wild birds, in backyard chickens related and not related with the social program Pro Huerta from INTA (Instituto Nacional de Tecnologia Agropecuaria), layers, layers breeders, broilers and broilers breeders and compare them. However, Recently, CAV genome was detected in commercially available poultry vaccines including HVT and Rispens in Brazil (18). The possibility of its presence in vaccines used in Argentina is seriously considered.

MATERIALS AND METHODS

Experimental design. Sera were obtained from the field and two types of backyard poultry populations randomly sampled and wild birds that either arrived to the Club de Observadores de Aves (COA) "Divisadero", "Grupo de Amantes de la Naturaleza del Partido de Pinamar (GANPP)" or were obtained by the author in different circumstances from places in the province of Buenos Aires. Field sera were obtained from samples submitted directly. Flocks ranged from 1 to 78 weeks of age and included layer-type and broiler-type parent flocks as well as layer and broiler flocks:

a) Four hundred sera were obtained from chickens which were part of a social program for food security Pro Huerta.

b) A second group of 350 sera were collected from backyard chickens from locations such as General Lamadrid, Laprida, General Madariaga, Paraje Juancho, City Bell, Gonnet y La Plata in the province of Buenos Aires. All donor flocks from these samples were far from commercial poultry operations.

c) A third group of 55 sera were obtained from different species of wild birds from different locations of the province of Buenos Aires.

d) A forth group of 345 individual sera examined from broilers breeders and broilers, as well as layer breeders and layers from the provinces of Buenos Aires and Entre Rios.

e) One SPF (Specific Pathogen Free) chicken flock.

The samples mention in A and 300 samples of B were tested at a 1:100 dilution using the commercial IDEXX enzyme linked immunosorbant assay (ELISA) CIAV antibody test kit. Samples of the second group were double checked using the indirect fluorescence antibody (IFA) test reported elsewhere (16, 23) and used essentially as described previously (4). Samples from wild birds (Group C) were obtained from the district of Pinamar, General Lamadrid, City Bell and Gonnet. They were only tested using IFA as 50 samples from Group B and all Groups D and E.

Cell lines. MDCC-MSB-1 cells were obtained from Dr Takeshi Mikami (at that time at the Department of Veterinary Microbiology, Faculty of Agriculture. The University of Tokyo, Tokyo, Japan) and from the Department of Avian and Aquatic Animal Medicine (DAAAM), Cornell University (CU), U.S.A. Both MSB-1 cell lines were grown at 39ºC or 41°C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 5% fetal bovine serum or in LM Hahn medium with 10% fetal bovine serum. The cells were sub cultured at 2 to 3 day intervals and seeded at 0.5 X 10^6/mL. More MSB-1 cells were culture as above, but at the Department of Microbiology and Immunology, CU during 2010. Smears were prepared with the virus described below and as mention also below.

Virus strains. The Cux-1 strain of CIAV (20) was obtained, with permission from the USDA by the DAAAM from Mr. R. Wellenstein (SPAFAS. Inc.,Norwich, Connecticut), after an unknown number of passages in MSB-1 cells. The same strain was obtained with permission of the Argentinean Ministerio de Economía y Obras y Servicios Públicos, Secretaria de Agricultura Ganadería y Pesca, Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) from DAAAM. Cux-1 CIAV was used to infect MSB-1 cells for the IFA test as described by McNulty *et al*. (16).

Monoclonal antibodies. Supernatant fluids from hybridoma 51.3 were used to detect the presence of CIAV-specific antigens. This hybridoma produces monoclonal antibodies (MAb) against the Cux-l strain of CIAV and cross-reacts with at least 2 other CIAV isolated in the USA (9).

Detection of antibodies against CIAV by IFA. Cux-1- infected and uninfected control MSB-1 cells were used as positive - and negative- antigen preparations for detection of antibodies. These were prepared as described by Lucio *et al*. (14) with a few modifications (4). Briefly, MSB-1 cells infected with Cux-1 were harvested at 48 hours postinoculation (PI) and washed three times with PBS. Ten uL containing 50.000 cells was placed in each well of a Teflon-covered glass microscope slide. Uninfected control smears were prepared with the same amount of uninfected MSB-1 cells on the same slide. The slides were air dried at room temperature, fixed in acetone for 10 minutes at room temperature, and stored at -20ºC. Ten uL of chicken serum, diluted 1:200 in PBS, were placed in CIAV-infected cells and uninfected cells: MAb 51.3 and PBS, were used as controls with each slide. After 30 minutes of incubation at 37ºC in a humid chamber, the slides were washed in PBS for 10 minutes at room temperature. Fluorescein isothiocyanate (FITC)- conjugated rabbit anti-mouse IgG and FITC- conjugated rabbit anti-chicken IgG were used at dilutions that did not cause fluorescence with CIAV- infected MSB-1 cells in the absence in CIAV-specific antibodies; for this positive and normal or negative serum provided by D. B. Lucio was used. Ten uL of the appropriate FITC-conjugated was then applied to the cells and incubated at 37ºC for 30 minutes, washed in PBS for 10 minutes at room temperature and mounted with Tris-glycerol (pH 8.5). Samples were read as positive only when clear granular fluorescence was observed in the nuclei of CIA-infected MSB-1 cells and fluorescence was not observed in the uninfected cell. The flock was considered positive only if more than five of 10 samples were found positive.

RESULTS AND DISCUSSION

Detection of antibodies to CIA in chicken sera. Antibodies to CIA were found to be widespread in sera from broiler breeders and layers throughout Argentina. Antibodies against CIA were found in 327 of 345 (95%) individual sera examined from broiler breeders and broilers, as well as layer breeders and layers from the provinces of Buenos Aires and Entre Rios. One SPF chicken flock tested had no antibodies to CIA.

This study has shown that antibodies to CIA are widespread in the field in chickens from both broiler breeders and layer parents, as well as commercial flocks, at different ages. Antibodies to CIA were not detected in an SPF chicken flock. Other studies provided evidence of the presence of antibodies against CIA in the poultry industry in many countries, but its economic significance is still unclear. The reason why the IFA test was chosen for

this serologic survey was that according to Yuasa et al. (22) the IFA test had the sane sensitivity as the neutralization test for detecting antibody against CIA and could be performed more quickly and easily. ELISAs which can be automated, was rapid and objective, but are still subject to the problem of non-specific reactions which are not necessarily overcome by the use of negative antigen.

In Argentina both heavy meat type birds and light egg-laying strains are infected; isolation of CIAV was first described in 1991 and since then the presence of CIA has been sporadic. However, a new isolation was reported recently, but the chickens from which the new isolate was obtained, came from eggs that had been imported from Europe. Perhaps, the proportion of vertically infected chickens is higher in countries where breeder flocks are not kept in close proximity.

None of the sera from the third and second group were positive in the ELISA test while 91% of the birds from the first group were positive with high titers. Sera from the second group, which were negative in the ELISA test, were also negative using the IFA.

The main differences between the two types of backyard birds sampled are their genetic background and the vaccines they received. Unlike the backyard flocks in the second group, the social program chickens were vaccinated with herpes virus of turkey (HVT) at the first day of age. The possibility of a contamination of the HVT vaccine has to be considered since recently a CIAV has been described as a contaminant of poultry vaccines (18).

Non-commercial birds in Europe had shown the presence of CAIV antibodies (11, 21). The first report of antibodies detected in non- commercial poultry and especially in backyard poultry in the Americas was published in 2006 (12) and the second was presented at the IXth Argentinean Virology Congress (3). Although Craig *et al*. (10) stated that no further studies on CAIV in Argentina were reported after 1994, information mentioned in the introduction shows the contrary (1, 2, 3, 7). Either the source of chickens used for the social program had CIAV or the environment in which they were kept was contaminated. A third cause could be that the HVT vaccine was contaminated with CIAV. Perhaps the mistake would have been to vaccinate those birds for Marek's Disease since the risk of introducing diseases with the use of not inactivated vaccines to birds that live in a free environment should be considered. Hernandez-Divers *et al* (13) pointed out that pathogens of free-ranging chickens create a risk of disease for wild birds. The probable harm done perhaps to the free living birds and chicken population in Argentina is potentially serious because CIAV can be spread among others birds since the chickens given by this social program could be apparently a source of virus. CIAV was probably introduced by commercial poultry operations. However, the role of the social program flocks as a reservoir for CIAV should be considered. However, preliminary studies conducted specially on free living wild birds, since they may also be reservoir for the virus transmission and so pose a threat to backyard and commercial poultry did not show positive data. The need for further studies should be considered since CIAV has already been recognized in wild birds in Europe (8). Means of control and biosecurity measures should be a high priority for poultry farmers and veterinarians. It is highly recommended that sanitary measures to eradicate and eliminate the disease from those birds if it exists should be applied. None of these chicken flocks are checked for the presence of infectious pathogens with the exception of Avian Influenza (AI) and Newcastle disease (7) and thus their health status is unknown. The Social program flocks that helped to understand the absence of AI in Argentina (6) may also be a reservoir for other avian diseases that could cause an economic and environmental threat, not only to the commercial poultry industry but to other backyard birds and potentially free living birds as well.

The use of live vaccines should not be recommended because the contamination of specific pathogens free (SPF) flocks with infectious pathogens as shown elsewhere (18) from where the vaccines are prepared is a possibility. There is a lack of facilities of SPF flocks in the world at the moment.

CONCLUSIONS

None of the sera from the second and third group were positive in the ELISA test while 91% of the birds from the first group were positive with high titers. All sera from the second group tested by ELISA or IFA resulted negative. The sera from wild birds up to the moment were negative to the IFA test as well as the sera from backyard poultry unrelated to the social program. However, further studies should be conducted specially on more free living wild birds in the surrounded of backyard hens vaccinated against MD since they may also be reservoir for the virus transmission and so pose a potential threat to the environment as well.

The need of more SPF farms was mentioned by Renato Verdi during the ninth "Jornadas Internacionales de Veterinaria Practica", in Mar del Plata, August 2015. He also mentioned that the high demand and the lack of enough SPF eggs make them not really SPF since sometime they are not properly checked and use for vaccine production anyway. These findings provide evidence for the possibility of contamination of poultry MD vaccines with CAV. It is emphasized the need of searching for these agents in all vaccines in order to ensure the absence of such potential contaminants. In the meantime, social programs such as the one mentioned in the

present work should evaluate consequences before being implemented. No vaccination with live vaccines should be recommended in backyard poultry at the moment and more flocks of SPF birds for vaccines development should be considered around the world.

ACKNOWLEDGMENTS

Mr Francisco Javier Crosetti Buscaglia for his unconditional help in many aspect of the preparation of this manuscript.

REFERENCES

1. Buscaglia, C., Un nuevo caso de anemia infecciosa de los pollos. In: Libro Resúmenes. V Congreso Argentino de Virología. II Encuentro Virólogos Latinoamericanos. Tandil, Argentina. pp 97. 1996.

2. Buscaglia, C., Anemia infecciosa de los pollos. In: Programa de Actividades y Resúmenes. VI Congreso Argentino de Virología. Mesa Redonda X: "Nuevas Prioridades en Sanidad Animal", Buenos Aires, Argentina. pp 27-29. 1999.

3. Buscaglia C. Presencia de anemia infecciosa de los pollos en aves del programa Pro Huerta. IX Congreso Argentino de Virología, Resumen 226-23798. Rev. Argent Microbiol 40:108-109. 2008.

4. Buscaglia, C. Estudio serológico preliminar de anemia infecciosa de los pollos en aves de traspatio de la provincia de Buenos Aires, Argentina. In: III Jornadas y Reunión anual de la Asociación Argentina de Inmunología Veterinaria. 1-2 November. Buenos Aires. pp 12. 2010.

4. Buscaglia, C., and Y. Tohya, Serologic survey for antibody against chicken infectious anemia virus in Argentina. In: Proceedings of the 46th WPDC, Sacramento, CA, USA. pp. 32-33. 1997.

5. Buscaglia, C., C.F. Crosetti, and P. Nervi. Identification of chicken infectious anaemia, isolation of the virus and reproduction of the disease in Argentina. Avian Pathol. 23:297-304. 1994.

6. Buscaglia C., C. Espinoza, M.V. Terrera, R. De Benedetti. Avian Influenza surveillance in backyard poultry in Argentina. Avian Dis. 51:467-469. 2007.

7. Buscaglia, C., M.V. Prío, M.C. Villat, V. Rodríguez, M.A. Risso, and G. Albo. Serological survey for Newcastle disease in non commercial layers that belong to a social program from Argentina called "Pro-Huerta". In: Proceedings of the 51st WPDC, Sacramento, CA, USA. pp. 81-82. 2003.

8. Campbell, G. Investigation into evidence of exposure to infectious bursal disease virus (IBDV) and chick infectious anaemia virus (CIAV) in wild birds in Ireland. In: Proceedings of the Second International Symposium on IBD and CIA. Rauischholzhausen, Germany. pp 230-233. 2001.

9. Chandratilleke, D., P. O'Connell and K.A. Schat. Identification of chicken anemia virus proteins with monoclonal antibodies. Avian Dis. 35:854-862. 1991.

10. Craig, M.I., A. Rimondi, M. Delamer, P. Sansalone, G.A. Knig, A.D. Vagnozzi, and A.J. Pereda. Molecular characterization of chicken infectious anaemia virus circulating in Argentina during 2007. Avian Dis. 53:331-335. 2009.

11. de Wit, J.J., J.H. van Eck, R.P. Crooijmans, A. Pijpers. A serological survey for pathogens in old fancy chicken breeds in central and eastern part of The Netherlands. Tijdschrift voor Diergeneeskunde 129:324-327. 2004

12. Hernandez-Divers, S.M., P. Villegas, F. Prieto, J.C. Unda, N. Stedman, B. Ritchie. Survey of selected avian pathogens of backyard poultry in northwestern Ecuador. J. of Avian Med. 20:147-158. 2006.

13. Hernandez-Divers, S.M., P. Villegas, C. Jimenez, S.J. Hernandez-Divers, M. Garcia, S.M. Riblet. Backyard chicken flocks pose a disease risk for neotropi birds in Costa Rica. Avian Dis. 52: 558-566. 2008.

14. Lucio, B., K.A. Schat, H.L. Shivaprasad. Identification of the chicken anemia agent, reproduction of the disease, and serological survey in the United States. Avian Dis. 34:146-153. 1990.

15. Lucio, B., , K.A. Schat, and S. Taylor. Direct binding of protein A, protenin G, and anti IgG conjugates to chicken infectious anemia virus. Avian Dis. 35:180-185. 1991.

16. McNulty, M.S., T.J. Connor, F. McNeilly, D. Spackman. Chicken anemia agent in the United States: Isolation of the virus and detection of antibody in broiler breeder flocks. Avian Dis. 33:691-694. 1989.

17. Michalski, W. P., D. O' Rourke, and T.J. Bagust. Chicken anaemia virus antibody ELISA: problems with non-specific reactions. Avian Pathol. 25:245-254. 1996.

18. Murtele Varela, A.P., H.F. Fernandes dos Santos, S.P. Cibulski, C. Mengue Schaffer, C. Schmidt, F.E. Sales Lima, A. D'Avila Silva, P.A. Esteves, A.C. Franco, P.M. Roehe. Chicken anemia virus and avian gyrovirus 2 as contaminants in poultry vaccines. Biologicals. 42: 346-350. 2014.

19. Schat, K.A., and V.L. van Santen. Chicken infectious anemia. In: Swayne, D.E., Glisson, J.R., McDougald, L.R., Nolan, L.K., Suarez D.L., Venugopal, N., (eds). Diseases of Poultry, 13th ed. Wiley-Blackwell. pp.249-264. 2013.

Verdi, R. Actualidades en la inmunización contra la enfermedad de Marek y desarrollo de la vacunación de embriones. 9th "Jornadas Internacionales de Veterinaria Practica", in Mar del Plata, 29th August 2015

20. von Bulow, V., B. Fuchs, E. Vielitz, and H. Landgraf. Early mortality syndrome of chickens after dual infection with Marek's disease virus (MDV) and chicken anemia agent (CAA). Zbl. Vet. Med. B 30:742-750.1983.

21. Wunderwald, C., and R.K. Hoop. Serological monitoring of 40 Swiss fancy breed poultry flocks. Avian Pathol. 31:157-162. 2002.

22. Yuasa, N., T. Taniguchi, and I. Yoshida. Isolation and some characteristics of an agent inducing anemia in chicks. Avian Dis 23:366-385. 1979.

23. Yuasa, N., K. Imai, and H. Tezuka. Survey of antibody against chicken anemia agent (CAA) by an indirect immunofluorescent antibody technique in breeder flocks in Japan. Avian Pathol. 14:521-530. 1985.

24. Zhou, W.B. Yang, B. Shen, S. Han and J. Zhou. A serologic survey of antibody against chicken infectious anemia virus by indirect immunofluorescent assay in domestic poultry in China. Avian Dis 40:358-360. 1996.

THE UPDATED REQUIREMENTS FOR ADDING ANTIMICROBIALS TO FEEDS

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OUTLINE

What would the world look like if we had responsible use of antimicrobials and how would the veterinarian be involved?

Bacteria develop resistance by four recognized pathways:

1. Darwinism selection
2. Gene transfer
3. Rupture and healing of the DNA (time related)
4. Unknown

There are numerous options under discussion and proposed regulations to reduce the agriculture use of antimicrobials. Will it be a top down regulation or a bottom up intergraded microbial management implementation plan? Currently most proposals have not gotten to regulation enforcement.

1. Veterinary Feed Directive of 2017.
2. California Senate Bill 27 (SB 27).
3. No Antibiotics Ever.
4. Certified Responsible Antibiotic Usage.
5. No antibiotics that are commonly used for humans.
6. Numerous variations of less antibiotics usage toward the end of the production period.

Emerging technology takes over 50 years to filter down to the veterinary field. Some that have not been used in the clinical setting in veterinary medicine are as follows:

1. Micro-biome management: there are numerous medical school departments exploring this technology. It has specific applications for gastro intestinal pathogens.
2. Whole Gene Sequencing (WGS): USDA is using this for environmental pathogen detection. It is more cost-effective than the traditional culture and sensitivity.
3. Bacterial Phages acceptance by the industry has not been very successful. It has a similar outcome as food irradiation technology for some of the same reasons.

Assessment with a plan for the need of antimicrobials and monitoring its expected outcome is our intent in public health. The HACCP principle application is needed and it should be documented in a expanded SOAP format. How do we implement this?

There are twelve things you should do and document before prescribing an antimicrobial. This is an Integrated Pathogen Management Plan:

1. Protocol or guidelines for each common disease syndrome in the clinical practice that might require antimicrobial intervention. These should also include #2 and #3 information
2. References to back up protocol.
3. Date protocol was up dated and reviewed. Managing pathogens requires continuous changes because of different local conditions and new findings.
4. Subjective information and chief complaint, including details of syndrome and history of medicine is most important.
5. Objective information observed by the clinician. If diagnostic test were performed the results are included.
6. Assessment and diagnosis (DX) usually selected from the protocol. If more than one condition is listed, they should be in order of priority.
7. The identified pathogens and their significance.
8. Plans with various options for intervention including information listed under # 9, 10 and 11. This should include any other testing to be performed
9. Prescribing the drugs (RX): This may require compliance with the Veterinary Feed Directive of 2017. In some settings, a copy of label would be appropriate as is often done in companion animal practice.
10. Follow up (F/U): There are four options, (a) veterinarian reviews the progress, (b) client contacts veterinarian with added information, (c) none needed unless progress is not as expected or (d) other. Usually there will be a specific predetermined date for the action and this should be included with a reminder for action notice.
11. Notes in didactic form. There is often needs and desires to add more information, with special instructions to client, clarification of information presented or clinicians' specific concerns including expected outcome. In this area, other options can be discussed. Information that may be relevant that has

not been included such as expected feed and water consumption can be included.

12. Reviewer's qualifications, including license number, date reviewed and contact information.

When will the profession charge a full fee for these services instead of getting paid from the sale of these drugs? Can the profession self-monitor?

REFERENCES

Bauer, Svars, Leopold, Levine, Whiter, (USDA, FSIS Working Group), 2016 White Paper Current Future Developments of Use of Modular Sub typing by USDA,FSIS, Internet pp 113

Blaser, Martin. 2014. The Missing Microbes: How over use of antibiotics is fueling our modern plague, Academic Press, NY.

Hoelzer, K. 2016. Antibiotic use in Food Animals: A growing threat to public health,

Food Safety Magazine, Dec 2016-January 2017. 30- 68.

U.S. National Research Council, 1999. The Use of Drugs in Food Animals: Benefits and risks, National Academy Press, Washington D. C.

USING SOCIAL NETWORK ANALYSIS TO IDENTIFY BACKYARD POULTRY STAKEHOLDERS IN CALIFORNIA

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ABSTRACT

Attendees of two Train the Trainers poultry workshops held in California were given a survey that asked them to list contacts that they collaborated with on backyard poultry (BYP) related work. In order to help identify current methods of knowledge dissemination among BYP enthusiasts, the surveys were analyzed using Social Network Analysis (SNA), which allows for a quantitative analysis of relationships among various stakeholders. While the SNA showed that the existing BYP network is decentralized and therefore not conducive for knowledge sharing and collaboration, key insights that could help restructure and improve the network were identified. As an example, among different poultry groups, 4-H was identified as the organization with the second highest coverage and fourth highest mean centrality score with respect to contact with BYP enthusiasts. This novel network-smart approach offers a more structured and targeted approach toward outreach efforts to backyard poultry enthusiasts in California.

INTRODUCTION

Backyard poultry ownership is undergoing an unprecedented level of growth nationally and in California. According to a 2010 APHIS-NAHMS study that survey, 1.2% of participating households in Los Angeles had backyard chickens and 4.6% more households planned to have backyard chickens within the next five years (5). At the same time, California's resources to educate BYP owners are limited. For instance, the number of UC Cooperative Extension (UCCE) specialists and advisers have drastically decreased due to significant budget cuts (1, 2). Specifically, with respect to poultry resources in California, there are currently only two poultry specialist and no poultry farm advisers. Therefore, maximizing resources in a targeted fashion is essential. One approach is to use social network analysis (SNA) which is a scientific and quantitative way of studying relationships to come up with visual and quantitative representations of how different stakeholders interface with one another (3, 4). It is well-known that social learning (i.e. learning through observation of others) is an important learning pathway in the agricultural field (3). Likewise, understanding the existing structure of the BYP network by identifying stakeholders and connecting BYP enthusiasts with BYP stakeholders can improve the dissemination of accurate information. For this reason, the California Department of Food and Agriculture (CDFA) sponsored two, two-day Train the Trainer Poultry workshops in two locations: Davis and Los Angeles, California. Attendees of these workshops received a survey asking them to list collaborators they frequently contacted with poultry related questions. The goal of the survey was to identify stakeholders and super spreaders of information. Once identified, outreach professionals can leverage stakeholder connections for quicker dissemination of information (i.e. notify BYP enthusiasts of a disease outbreak) and to promote valuable resources that are available to backyard poultry enthusiasts.

MATERIALS AND METHODS

The target audience for the meetings included various poultry stakeholders. This served two purposes: disseminated the information presented at the meetings more efficiently and building a "snapshot" of the BYP network that was more representative and connected than if it were random BYP enthusiasts participating. Feed store managers, club leaders (i.e. Meetup organizer), government affiliates (i.e. CDFA, UC ANR), veterinarians, BYP enthusiasts and farm owners were considered stakeholders because of their potential leadership role

in the poultry community meaning that they are vital actors in the dissemination of information.

The collaboration network section of the survey asked respondents to list up to eight individuals they collaborated with on BYP related work. This paper will look only at the results from the collaboration section.

Using the contacts listed in the collaboration section of the survey, a relational matrix was built. The matrix was then analyzed and visualized using the SNA software ORA. The collaboration network is made up of nodes representing individuals and ties representing collaboration relationships. For the purposes of this study, collaboration networks can also be called knowledge-sharing ties.

RESULTS AND DISCUSSION

Surveys were given to all the attendees and filled out either in paper form or online at the end of both meetings. 53 out of 67 attendees completed the survey for an overall response rate of 79%.

Figure 1 shows that the BYP collaboration network is decentralized with the majority of the clusters (22 independent clusters) having a relatively low density, meaning that few nodes are connected to each other (4). This decentralized structure means that there is potential for improvement with intervention when it comes to collaboration and knowledge sharing (6). Additionally, Figure 1 highlights the network position of those individuals associated with some key institutions. An important observation here is that CDFA makes up a majority of the network's largest cluster, and seems to collaborate with other important institutions including UCCE, UCD researchers, and some other trainers. However, the network also indicates that CDFA could improve at collaborating with other important network actors, especially 4-H leaders (Figure 1). 4-H members seem to be widely dispersed across ten collaboration network clusters (Figure 1). This suggests that the institution is well-positioned for collaboration and information sharing. CDFA and UCCE would benefit from strengthening relationships with 4-H.

Total degree centrality is an individual-level measurement that counts the number of ties a node has (4). As shown in light gray in Table 2, individuals with job types having the highest mean centrality were BYP group association members (3.25 collaboration relationships on average), county ag commissioners (3), BYP practitioners (2.79), 4H leaders (2.68), and CDFA staff (2.67). These results suggest that individuals with these job types are well-positioned in the network to act as collaboration leaders. In contrast to centrality, coverage is a population-level measurement that gives insight as to how many collaborators each professional group can potentially connect with (4). The stakeholders with the highest coverage were BYP practitioners (coverage of 120), 4H leaders (67), CDFA (40), BYP group association members (39), and feed stores (24) as seen in Table 2 with the top five highlighted in dark gray. These results suggests that these job type populations with the highest centrality (individual-level) and coverage (population-level measurement) are well positioned in the network to act as stakeholders because of how central they are to the network (3). Such individuals have the greatest potential to be aware of others projects because they are in touch with many others in the backyard paltry community. They are probably able to rapidly coordinate new projects, establish partnerships, and mobilize assets because they are connected to others who themselves are connected to many others who have access to additional or different resources (6).

CONCLUSION

The collaboration network provides insight into how UCCE and CDFA can leverage the network to optimize collaboration and knowledge sharing among BYP practitioners and trainers. UCCE and CDFA should focus on building new or strengthening existing relationships with those institutions that were found to have high measures of coverage. Working with those institutions who were found to have low measures of centrality and coverage to boost their standing in this regard would help strengthen the network's capacity for collaboration and knowledge sharing.

More specifically, outreach partnerships with 4H should be prioritized. 4H leaders were found to be extremely well connected in the collaboration network (Figure 1). Therefore, efforts should be made to direct outreach through these "super-spreaders" of information. By reaching out to 4-H, CDFA, UCCE and SVM would significantly increase their social network. Continual usage of SNA will allow further optimization of a network-smart approach to outreach in order to maximize knowledge sharing and collaboration among various BYP enthusiasts.

(The full-length article will be submitted to the *Journal of Preventive Veterinary Medicine*.)

REFERENCES

1. Cline, H. 2003. UC Cooperative Extension cuts have begun. *Western Farm Press.*

2. Fruit Grower News. 2007. UC Cooperative Extension Battles Back From Drastic State Budget Cuts. *Fruit Grower News.*

3. Hoffman, M., M. Lubell, and V. Hillis. 2015. Network-smart extension could catalyze social learning. *California Agriculture*. 69:113-122.
4. Knoke, D., and S. Yang. 2008. Social network analysis. Sage.
5. NAHMS. 2013. Urban Chicken Ownership in Four U.S. Cities.
6. Valente, T. W. 2012. Network Interventions. *Science*. 337:49.

Figure1. BYP network with nodes representing individuals associated with some key institutions and linkages representing collaboration ties.

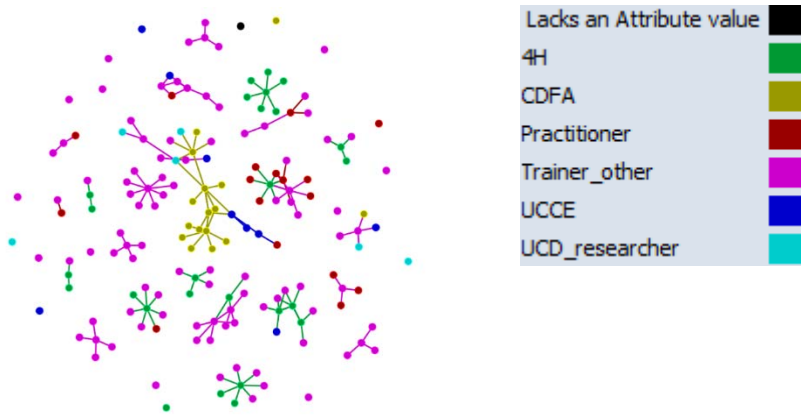

Table 1. Mean total degree centrality, centrality rank (highligted in light gray), population size, coverage and coverage rank (highlited in dark gray) for stakeholder groups.

	Mean degree centrality	Centrality rank	population size (*n*)	Coverage	Coverage rank
BYP practitioners	2.79	3	43	119.97	1
4-H	2.68	4	25	67	2
CDFA	2.67	5	15	40.05	3
BYP group association member	3.25	1	12	39	4
Feed store	1.85	9	13	24.05	5
Veterinarian	1.55	10	11	17.05	6
Breeder, hatchery, pullets producer	1.5	11	10	15	7
Egg/Broiler producer	2.17	7	6	13.02	8
County ag commisioner	3	2	4	12	9
Community farm garden	2	8	6	12	10
Friend/Family	1	14	10	10	11
UCCE advisor	1.45	13	5	7.25	12
UCD researcher	1.5	12	4	6	13
UCCE specialist	2.5	6	2	5	14
Government other	1	15	3	3	15
APA	1	16	2	2	16
FFA	1	17	2	2	17
Master gardener	1	18	2	2	18
ASPCA	1	19	1	1	19

VACCINATION WITH SIDEROPHORE RECEPTORS AND PORINS PROTECTS AGAINST FOWL CHOLERA CHALLENGE BY HETEROLOGOUS SEROTYPES

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SUMMARY

Fowl cholera is a contagious lethal disease affecting both wild and domestic fowl caused by *Pasteurella multocida*. Conventional vaccines are widely used to protect commercial poultry flocks. For typical commercial bacterins to be effective, whole cells of one to four common serotypes are included. These bacterins typically only protect against serotypes included in the vaccine. Autogenous whole-cell bacterins may also be used to protect against serotypes not commonly found in commercial vaccines.

Epitopix has developed a new fowl cholera vaccine using the company's Siderophore Receptor and Porin (SRP®) technology. The vaccine was produced using a serotype 3x4, and a serotype 2x5 strain of *Pasteurella multocida,* which when combined were identified to contain the appropriate SRP composition. Birds were vaccinated with Pasteurella SRP, divided into two groups of vaccinates and placebo control chickens and challenged IM with either *Pasteurella multocida*, a serotype (7, 9, 10) strain, or a serotype (8, 14, 15) strain. Results of these Challenges gave 92% and 70% prevention of mortality, respectively, in vaccinates compared with control birds. This study conclusively shows cross serotype protection using a SRP technology based *Pasteurella multocida* vaccine.

BACKGROUND

Historically, commercial inactivated *Pasteurella multocida* vaccines have based their protective immune response on O-polysaccharide associated with each strain's serotype. Unfortunately, vaccines based on this strategy are serotype specific and thus only confer protection against the serotypes included in the vaccine. Rather than basing our vaccine on O-polysaccharide, Epitopix SRP Pasteurella vaccine's protective response is based on iron acquisition outer membrane proteins and porins.

The ability to survive and proliferate successfully within its host is a necessary characteristic of any bacterial pathogen. The host defends against bacterial invasion by creating a hostile environment for the bacterium, which includes conditions such as limited oxygen availability, osmotic and pH stress, the presence of antimicrobial enzymes, and nutrient restriction. Many of these obstacles are overcome by the protective role of the major porin proteins located in the outer membrane of gram negative bacteria that allow solute diffusion or mediate active transport. The four major groups of porins are the general porins; the monomeric porins; the specific porins; and the TonB-dependent, gated porins (iron-siderophore/hemophore receptor proteins). The various porin types allow pathogens to grow in diverse environments. For example, iron is a primary nutrient required by most bacteria for electron transport and for cofactors for essential metabolic enzymes. Iron is severely limited during microbial invasion of a host species; the host forms iron complexes with high affinity, iron-binding proteins such as transferrin in blood, lactoferrin in secretory fluids, ovotransferrin in albumin, and ferritin within cells. The low availability of iron within a host is a barrier to infection that microorganisms must overcome in order to proliferate. Bacterial pathogens have developed strategies for obtaining iron from their hosts. A common method bacteria employ to acquire iron is the production of receptors in the outer membrane which bind the host iron-bound molecules and interact with other membrane-associated proteins to internalize the iron. These receptors and other porins, collectively referred to as the SRPs, are surface exposed, highly conserved and expressed in high copy number on the outer membrane. These traits make SRPs good candidate antigens for cross protective vaccine development.

MATERIALS AND METHODS

General protocol. The vaccination/challenge protocol was a modified procedure adapted from 9CFR 113.117 potency test for Pasteurella Multocida Bacterin, Avian Isolate, Type 1(1).

Chickens. Specific Pathogen Free Leghorn chickens were obtained from Valo BioMedia, (Adel, IA) and grown to 12 weeks of age. The chickens were

divided into four groups of 21 with 10 females and 11 males in each group. Individual birds were identified by numbered wing bands. Band numbers were blocked by sex and randomized. All chickens were housed together and provided antibiotic-free feed and water ad libitum.

Vaccination. Chickens were vaccinated subcutaneously in the back of the neck at 12 weeks of age and again 21 days later. Groups received vaccine and challenge as listed in Table 1. Vaccinate groups received SRP vaccine prepared from a 3x4 serotype and a 2x5 serotype of *Pasteurella multocida*. Placebo groups received vaccine containing adjuvant and saline, but no vaccine antigens.

Challenge. The challenge organisms were recent field isolates causing disease in commercial poultry operations. The strains were confirmed Pasteurella multocida by PCR and identified to serotype by agar gel immunodiffusion test. Cultures were grown overnight at 37°C in trypticase soy broth and adjusted to an appropriate level of colony forming units (CFU) for challenge. Challenge counts were confirmed by serially diluting the culture and plating each dilution on TSA II 5% sheep blood agar plates, in duplicate. Twenty birds of each group were challenged in the breast muscle with 0.5 mL 14 days following the second vaccination. Extra birds were removed from the study at this time.

Observations. Chickens were observed for mortality daily for 7 days. Any moribund birds were euthanized per the Center for Veterinary Biologics Testing Protocol SAM 607 (2).

RESULTS

At a challenge dose of 5.0 x10^8 CFU, both field strains were highly virulent (50% mortality for serotype 7,9,10 and 65% mortality for serotype 8,14,15). Protected fraction was 70% against serotype 7x9x10, and 92% against serotype 8x14x15(Table 2). The two-sided p-value, as calculated by Fisher's Exact Test, was 0.04 for the 7x9x10 challenge, and 0.0001 for the 8x14x15 serotype challenge, indicating a significant and extremely significant difference, respectively.

DISCUSSION

Fowl cholera is common across the globe and is known to be transmitted by common birds and animals. Infected flocks can very quickly realize high mortality, as well as a significant decrease in productivity in layers and breeders. While treatment and removal of infected birds is required for control, strict biosecurity and vaccination programs are central to the prevention of large outbreaks of disease in commercial flocks.

There are generally two types of commercial vaccines for fowl cholera: killed bacterins and live attenuated vaccines. Attenuated live vaccines have short lived immunity so the need for multiple revaccinations is common. Attenuated live vaccines may also cause illness in some birds post-vaccination.

Previous research has been unable to demonstrate serotype cross protection against *P. multocida* by bacterins produced in iron-depleted medium (3). Producers often turn to autogenous killed vaccines in response to disease and death in flocks vaccinated with commercially available fowl cholera vaccines which, while they offer better protection for serotypes specific to the farm site, do not protect against serotypes that are not present in the vaccine.

The SRP technology has been used in several commercial poultry operations for over a decade in autogenous form to successfully control fowl cholera, and the technology has been validated to work under field conditions using the licensed bovine Salmonella Newport vaccine (4), and conditionally licensed bovine *Escherichia coli* Bacterial Extract vaccine (5) produced by Epitopix.

The present study provides an important demonstration that vaccine made with the SRP proteins of *Pasteurella multocida* provides broad protection against field strains of heterologous serotypes of virulent *Pasteurella multocida*. This vaccine has met efficacy requirements of the USDA Center for Veterinary Biologics against serotype 1 in chickens, and is currently in safety trials across the USA. This technology continues to show promise to protect against multiple serotypes of *Pasteurella multocida* in commercial poultry operations.

REFERENCES

1. US Department of Agriculture Animal and Plant Health Inspection Service. Pasteurella Multocida Bacterin, Avian Isolate, Type 1. Code of Federal Regulations, Title 9, Chapter 1 Part 113.117. 1991.

2. USDA Center for Veterinary Biologics. SAM 607.03: Supplemental Assay Method for Potency Testing of Fowl Cholera (*Pasteurella multocida*) Bacterins, Type 1. Supplemental Assay Methods. Ames, IA: USDA-APHIS-CVB, pp1-11. 2009.

3. Glisson, J.R., et al. Cross-Protection Studies with *Pasteurella multocida* Bacterins Prepared from Bacteria Propagated in Iron-Depleted Medium. Avian Diseases 37:1074-1079, 1993.

4. Hermesch, D. R., et al. Effects of commercially available vaccine against *Salmonella enterica* serotype Newport on milk production,

somatic cell count, and shedding of Salmonella organisms with no clinical signs of salmonellosis. Am J Vet Res. 69:1229-34. 2008.

5. Thomson, D. U., et al. Use of a siderophore receptor and porin proteins-based vaccine to control the burden of *Escherichia coli* O157 in feedlot cattle. Foodborne Pathog Dis. 6: 871-7. 2009.

Table 1. Treatment and challenge groups.

Group	Vaccine treatment	Challenge Serotype
A	SRP serotypes 2x5 and 3x4	7, 9, 10
B	Placebo	7, 9, 10
C	SRP serotypes 2x5 and 3x4	8, 14, 15
D	Placebo	8, 14, 15

Table 2. Mortality results of challenge.

7,9,10 Challenge	# Dead/ # Tested	% Mortality	Prevented Fraction
Vaccinate	3/20	15%	70%
Placebo	10/20	50%	
8,14,15 Challenge			
Vaccinates	1/20	5%	92%
Placebos	13/20	65%	

A COMPARISON OF 46-WEEK SEROLOGY AND IBV ARKANSAS PROTECTION IN FLOCKS RECEIVING DIFFERENT COMMERCIAL SE BACTERINS

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INTRODUCTION

The use of *Salmonella* Enteritidis (SE) bacterins is commonplace in the commercial egg layer industry. Typically, companies will take advantage of this vaccine handling by giving trivalents which also include Newcastle disease virus (NDV), and infectious bronchitis virus (IBV) antigens. Most of these trivalent vaccines contain only Massachusetts serotype IBV but at least one also contains an Arkansas serotype IBV. There is a concept (credited to Gelb and Killian, 1987) known as "original antigenic sin" which postulates that birds will continue to respond to the first IBV serotypes they are immunized against even if subsequent exposures are to other serotypes. The objective of this study was to compare the serological response and Ark protection of layers primed with a live Ark vaccine and later given SE-ND-IB vaccine containing either Mass only or both Mass and Ark IBV antigens.

MATERIALS AND METHODS

Commercially raised pullets were vaccinated with live vaccines B1/Mass-Ark at two weeks of age followed by B1/Mass-Conn at five weeks and LaSota/Mass-Holland at nine weeks. At 13 weeks of age, birds located in adjacent rows were vaccinated with either killed SE Vaccine A (contains only Mass) or Vaccine B (contains both Mass and Ark). At 15 weeks of age, birds from each treatment were leg banded and placed in adjacent cages on a layer farm. At 45 weeks of age, 40 birds from each treatment were transported to isolator facilities in Durham, N.C., where they were housed eight per randomized isolator (three challenge reps and two non-challenge reps). Thirty two-week old SPF leghorns were also housed 10 per isolator (two challenge and one control rep). After one week of acclimation, controls were bled and challenged birds received 4.5 logs (EID50) of Ark IBV by eye/nose drop. Serum was tested using SE, ND and IB kits. All birds were evaluated five days post-challenge for IBV protection based on clinical signs, tracheal histopathology and PCR of choanas and tracheas. Statistical analysis was not applied across vaccine treatments because pullets were only segregated by adjacent rows and not interspersed by cages.

RESULTS

Titers were very similar between Vaccine A and B (see Table 1). There were no significant clinical signs in the challenged groups, including SPF controls. 63% of challenge controls displayed three-scores (necrosis) on histopathology compared to 0% in the vaccine treatments. 83-100% of vaccinated birds, whether challenged or not, displayed two-scores (infiltrates, epithelial hyperplasia), which is considered normal in vaccinated, commercially raised birds. Vaccinate controls were negative for IBV based on quantitative PCR while SPF challenge controls were 100% positive on both choanal and tracheal samples. In contrast, Vaccines A and B were 79% and 67% PCR-negative on choanal samples and 83% and 96% PCR-negative on tracheal samples, respectively. Using a cut-off CT (cycle time) value of 30, Vaccines A and B showed 96% and 100% tracheal protection.

DISCUSSION

It was interesting that the SPF challenge controls did not show clinical signs, although they were 100% infected and about two-thirds showed tracheal necrosis from the Ark challenge. The complete lack of tracheal necrosis in the vaccine treatments attests to their solid immunity against the Ark challenge. SE, ND and IB serology results were very similar between the two vaccine treatments—even on Ark HI GMTs. On protection against recovery of Ark IBV challenge (PCR-negative), Vaccine A numbers were slightly higher on choanal protection (79% vs. 67%), while Vaccine B (containing Ark antigen) numbers were a little higher on tracheal protection (96% vs. 83%). While it is debatable whether the two-week-old live Ark vaccination provided an "original antigenic sin" effect for both inactivated SE

vaccines, this study demonstrated that both vaccination programs provided similar levels of Arkansas serology and protection in 46-week-old layers.

Table 1. IBV serology by vaccine treatment group (non-challenged controls).

		IBV Serology (GMT)				NDV Serology (GMT)			SE Serology
		ELISA		HI		ELISA		HI	ELISA
Treatment	IBV antigens in formulation	Idexx	Pro Flock	Mass	Ark	Idexx	Pro Flock	UGA	Biochek
SE Vaccine A	Massachusetts	6845	11032	830	596	8140	7738	1046	3497
SE Vaccine B	Massachusetts and Arkansas	7113	12372	932	538	6424	5440	1140	3220

AVIAN NEUTROPHILS –`A LA G. LESBOUYRIES, *PATHOLOGIE DES OISEUX* – 1941, A DEMONSTRATION WITH MATERIAL FROM DUCKS

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SUMMARY

A duck granulocyte of the heterophil series having cytology similar to "neutrophiles" of chickens earlier described by Lesbouyries (2) was in blood. Called "typical heterophil" (HT) its faintly stained fusiform granules, differentiate HT's from classic heterophils (HC) and a minority type "variant heterophil" (HV) with orange granules. HT's were the most common type (54%, 3803/7030) HC's (45%, 3136/7030) HV's (1%, 86/7030) in standard differential counts (SDC) of 6 wk ducks. Bone marrow smears of lame ducks also contained all three heterophils. As the ratio of heterophils to lymphocytes (H/L) is used as a stress measure, the existence of HT's and HV's, in addition to classic heterophils, affects interpretation of leukograms.

INTRODUCTION

As is the case with other avians, a duck granulocyte is categorized as, a heterophil, a basophil, or an eosinophil (3). The cytoplasmic granules of both heterophils and eosinophils are red, but usually shaped differently; nuclear configurations are additional distinctions. Basophils whose granules are deep purple metachromatic spheres of varying size are easily recognized. Although their granules are red (eosinophilic) and fusiform; many authors consider avian heterophils equivalent to mammalian neutrophils. However, Lucas (3) citing Lesbouyries (2) indicated the possibility of an avian neutrophil. Heterophil variation of ducks is the main subject of this manuscript. Its purpose is to demonstrate cells resembling Lesbouyries' neutrophils, called HT's, are common in blood and bone marrow. A secondary purpose is to draw attention to problems that multiple heterophil types impose on interpretation H/L ratios a method widely used to determine stress.

METHODS

Blood, bone marrow, stain procedures, and microscopy. Whole blood drawn from the leg veins, and femur bone marrow slides were stained using an in-house version of Wright's method followed by a brief secondary exposure to Giemsa. Olympus CX-41 light microscope with 100x oil objective; image capture with Infinity-2 1.4 megapixel CCD USB 2.0 Camera, were processed with Infinity Analyze software.

RESULTS

A higher frequency of HT's occurred in central slide locations than at edges ($P < 0.03$) but more medium sized lymphocytes ($P < 0.04$) and promyelocytes ($P < 0.01$) located to edges, indicating sorting (non-random distribution) tendencies. Total white cell counts (TWBC) of ~ 110 K/µL were clearly in the leukocytosis/leukemoid reaction range. Figure 1 (A) A granulocyte nest in duck bone marrow (22 wk) surrounding a developmental stage cell (promyelocyte, PM) are compared with chicken HC's described by Lucas. The numbers correspond to equivalent chicken cells given in Lucas (3). The two HT's at 12 and 4 o'clock resemble Lesbouryies "neutrophiles". The cytoplasm of the HT at the lower right with a slight blue tinge contains small clear zones (vacuoles) just outside the three lobed nucleus; the largest vacuole is at 5 o'clock. Vacuoles and blue cytoplasm represent toxicity. Several nearby erythrocytes with gray-blue hues are polychromatic or developmental cells often seen in bone marrow. (B) A blood HT (typical heterophil) resembling the Lesbouryies neutrophile and a classic heterophil (HC) is located nearby. Both cells have two lobed nuclei with condensed chromatin. The granulocytes appear on a background of polychromatic erythrocytes. (C)The classic heterophil (top) with a three lobed nucleus, the left member of which has disintegrated (karyorrhexis). The HC cytoplasm has deep red fusiform granules with faint suggestion of central bodies. The two HTs resemble the Lesbouryies neutrophile have three lobed nuclei connected by thin filaments. Both the cytoplasmic and nuclear membranes are intact. (D) HTs and an HC with nuclear lobe numbers indicated (number) appear along with a medium lymphocyte (Lm) and a thrombocyte (Th).

DISCUSSION

The light microscopic evidence presented suggests at least three distinct types of heterophils occur in the bone marrow and circulating blood of commercial ducks. HC similar to other avians compose only one type. A second heterophil resembling "neutrophiles" described in chickens by Lesbouryies (2) are another type. HTs were more common than HCs in the circulation at six weeks. HTs resemble mammalian neutrophils because of pale cytoplasmic granulation. HTs cannot be the result of dye dissolution as speculated by Lucas (2). This is because they occur in the same microscopic fields as fully stained HCs, other types of leukocytes, and erythrocytes. They display intact nuclear and cellular membranes a further indication their differences with HCs are biological. Most HTs do not display classic toxicity indicators as cytoplasmic vacuoles or toxic granules. Ducks, as do other avians, have a third heterophil distinct from both HC and HT. This cell (called a heterophil variant, HV) has cytoplasmic granule (orange spheres) and nuclear configuration differences with HCs and HTs. Thus, they represent a separate (third) granulocyte lineage. HCs, HTs and HVs occur in bone marrow (Figure 1 A) in the same microscopic field a further indication each has a distinct lineage.

The demonstration of multiple heterophil types contributes to basic hematology of ducks and has practical significance. This relates to the use of heterophil/lymphocyte ratio as a stress indicator. The demonstration of multiple heterophil types adds a complicating layer to the interpretation of a simple H/L. If either HTs or HVs represent novel forms of toxicity, a simple H/L will be insufficient as a stress determinant (1).

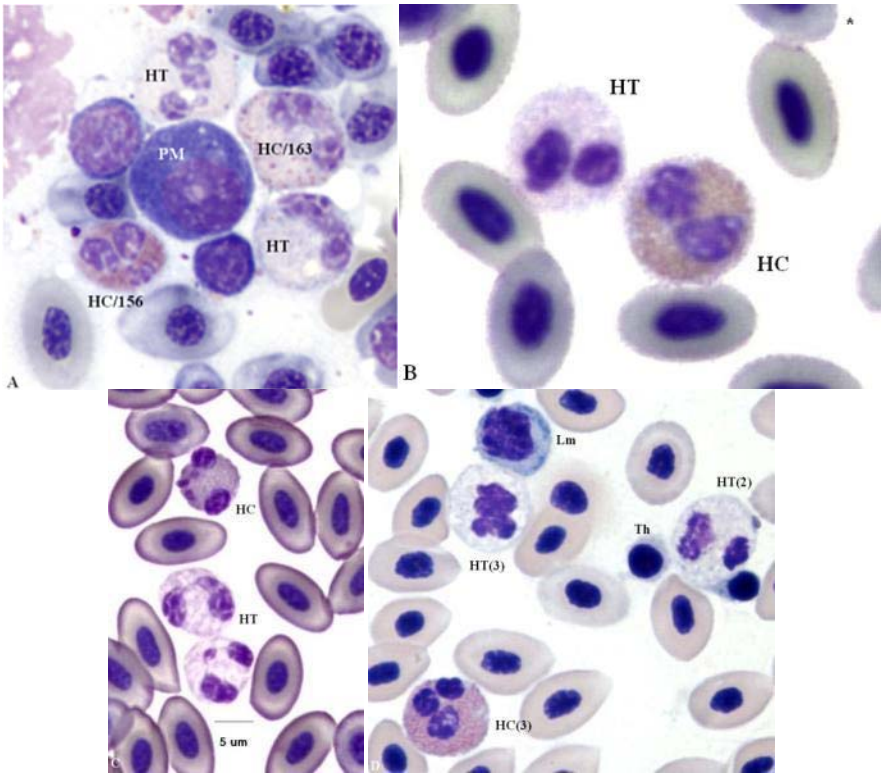
REFERENCES

1. Cotter, P. F. An examination of the utility of heterophil lymphocyte ratios in assessing stress of caged hens. Poult. Sci. 94:512–517. 2015.

2. Lesbouyries, G. La Pathologie des Oiseaux, Vigot Freres, Ed. Paris. 1941.

3. Lucas, A. M., and C. Jamroz, Atlas of Avian Hematology, Monograph 25 USDA, Washington, D.C. 1961.

Figure 1.



DUCK TÜRK CELL RESPONSE TO AFB1

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SUMMARY

Blood films drawn from 14 day male ducklings fed aflatoxin B1 and controls are described. Bacteremia and fungemia were common and hemograms were atypical. There was the high incidence of lymphoid cells having cytoplasm divided into deeply basophilic ectoplasm and endoplasm regions and fenestrated by clear vacuoles, sometimes with Hof's. Cell sizes varied from < 5 µm to > 19 µm in diameter, many with irregular margins. Their cytology suggests they are mitotic proplasmacytes, similar to Türk irritation cells (Reizungformen) of mammals. Cells at all stages of the division cycle were in both groups. In several instances, the chromosomal phases and cytokinesis were asynchronous as daughter cells began premature separation. As sensitivity to aflatoxins is known to be high in ducks these observations may assist in understanding how fungal metabolic products impacts performance.

INTRODUCTION

Hemograms (blood pictures) are useful in detecting disease. A derivative, the ratio of heterophils to lymphocytes (H/L) may inform as to stress, in its absence low H/L's are the norm; however, atypia may complicate interpretations (3). Here the Türk cell proplasmacyte is the subject. These deeply basophilic cells are highly variable in size, and may retain a capacity for mitosis has long been known in mammals (4). Blood obtained from 14 dy male ducklings as a part of an experiment on how aflatoxin B1 (AFB1) effects performance is the source. Türk cells were in blood of both experimental and control groups, likely a reaction to naturally occurring bacteremia, fungemia, and the aflatoxin treatment. Examples of Türk cells, a.k.a. "transitional cells" are presented in the company of plasmacytes and other atypical forms.

METHODS

Ducks, blood, diets, stain procedure, and microscopy. Whole blood was drawn at day 14 from the leg veins from a subset of 144 male Pekin ducklings housed in battery cages on wire floors. Diets contained AFB1 added at 0.0, 0.11, 0.14, 0.21 mg/Kg (1). Staining was by an in-house version of Wright's method followed by a brief secondary exposure to Giemsa. Photos were obtained with an Olympus CX-41 light microscope with 100x oil objective; image capture with Infinity-2 1.4 megapixel CCD USB 2.0 Camera. Photo processing was with Infinity Analyze software.

RESULTS

Examples of Türk cells, and other atypia, in the following figure are chosen to illustrate size variability, mitosis, and other unusual features.

Fig. 1 (A) is amedium size (~12 µm) Türk proplasmacyte (Lm) displays an eccentric nucleus and classic deep blue-purple cytoplasm fenestrated by clear vacuoles. Some vacuoles are organized into an extranuclear 'Hof' seen at 12 o'clock. Chromatin is coarse with three or four central nucleoli. A typical heterophil (HT) a small lymphocyte (Ls) and a polychromatic RBC (pRBC) are nearby, Bar = 5µm. (B) A basophil (left) displaying the characteristic metachromatic cytoplasm with granules contrasts with a medium size Türk cell having a large central nucleus and surrounded by a thin rim of deep blue cytoplasm with a few lighter blebs. (C) A large (D 19.25 µm, A 202 μm^2, P 55 µm, N/C ~ 0.7) early developmental Türk cell displays an irregular dark ectoplasmic edge with cytoplasm fenestrated by variously sized clear and slight pink vacuoles. Several nuclear vacuoles (Dutcher bodies) and faint nucleoli are seen against a background of fine chromatin. (D) Asynchronous anaphase and premature cytokinesis in a Türk cell with cytoplasmic clasmatosis.

DISCUSSION

Cells resembling classic Türk irritation cells (Reisungformen) were in the blood of ducklings fed aflatoxin AFB_1 and also in the control group. These proplasmacytes are "sentinel" cells indicative of a complex hemogram and so infer stress and pathology. As control and AFB_1 groups were Türk cell positive and had microscopic evidence of bacteremia and fungemia it is likely that they are a response to microbial substance. Because of their deep basophilia, size, shape irregularities and a mitotic capacity they are easily recognized by light microscopy. Türk cells may be related to Mott cells (atypical plasmacytes) frequently found in complex hemograms (2, 3). To the

author's knowledge this is the first report of Türk cells to appear in avian hematologic literature.

REFERENCES

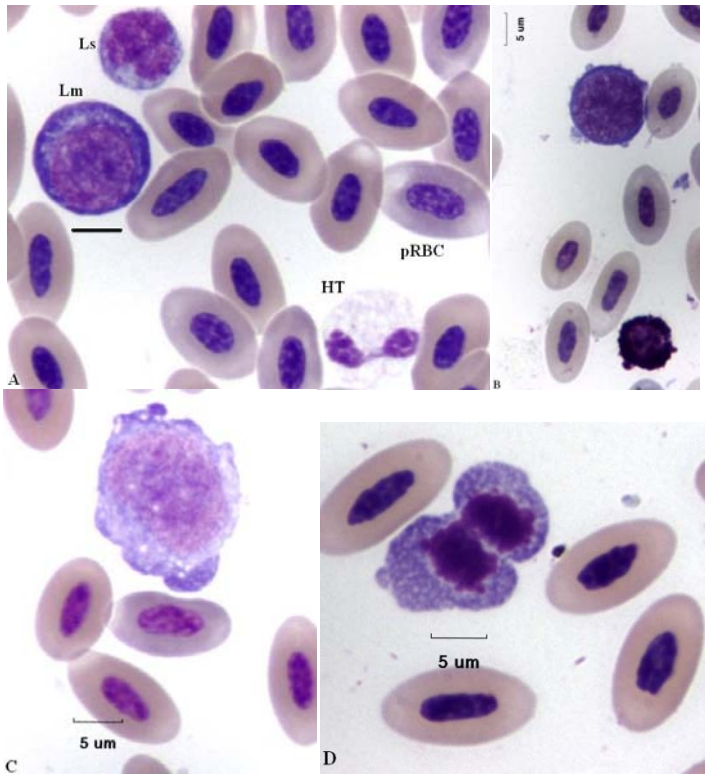
1. Chen, X., N. Horn, P. F. Cotter, and T. J. Applegate. Poult. Sci. 93:2028–2036, 2014.

2. Cotter, P. F. Are peripheral Mott cells an indication of stress or inefficient immunity? Poult. Sci. 94:1433–1438, 2015.

3. Cotter, P. F. and M. R. Bakst. A comparison of Mott cell morphology of three avian species. II. – Bad behavior by plasmacytes? 2016. Poult.Sci. 00:1–7 http://dx.doi.org/10.3382/ps/pew288, 2016.

4. Türk, W. (Ed.) In: Klinische Untersuchungen uber das verhalten des Blutes bei akuten Infektions Krankheiten. Braumuiller. Vienna, 1898.

Figure 1.



VACCINATION AGAINST *SALMONELLA ENTERICA* ENTERITIDIS USING SIDEROPHORE RECEPTOR AND PORIN PROTEINS

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SUMMARY

Salmonella remains a major food safety concern with poultry products. Whole-cell bacterin vaccines are widely used to protect commercial poultry flocks, but their effectiveness is limited by two factors. First, whole-cell bacterin vaccines include a wide range of proteins that are not immunoprotective. Second, each vaccine only protects against the specific serotypes included in its formulation. While autogenous vaccines can extend to cover serotypes not commonly found in commercial products, they are still limited to their source serotypes. In contrast, Siderophore receptor and porin-based (SRP®) vaccines are directed towards a much smaller set of proteins to focus the immune response against iron acquisition; a significant pathogenic mechanism of bacteria. Further, SRP's are highly homologous proteins and hold the promise of broad protection unlike those based on serotype or somatic antigens.

Willmar Poultry Company has a 20 year history of successful vaccination against *Salmonella* in breeder turkeys using a SRP vaccine and recently, Epitopix, a subsidiary company, has developed a new *Salmonella enterica* Enteritidis (SE) vaccine for use in chickens using the same technology. Vaccinated and placebo control chickens were challenged with SE and subsequent shedding and reproductive organ colonization were examined. Shedding was significantly reduced in vaccinates compared to controls and there was complete protection of the reproductive organs compared to the placebo group. This represents the first step towards an inactivated vaccine expected to be broadly protective for *Salmonella* species in chickens.

BACKGROUND

Iron is an essential cellular nutrient required for electron transport, thus energy production, and for enzymatic cofactors involved in essential metabolic processes. As such, iron acquisition is essential for gram-negative bacterial growth; a prerequisite for colonization and pathogenesis. However, iron is severely limited during microbial invasion of a host species because the host forms iron complexes with high affinity iron-binding proteins such as transferrin in blood or lactoferrin in secretory fluids that make iron generally unavailable. This lack of availability of iron within a host is a barrier to infection that microorganisms must overcome to proliferate. To counter this, bacterial pathogens have evolved specific molecules for obtaining iron from their hosts. Bacteria secrete small iron-chelating molecules, called siderophores, which bind iron with high affinity and "steal" iron directly from the host binding proteins. Bacterial porin proteins on the outer membrane are receptor proteins for iron-siderophore complexes and facilitate the internalization of iron. The siderophore receptors and other porins, collectively referred to as the SRPs, are surface exposed, highly conserved and expressed in high copy number on the outer membrane. These traits make SRP good antigens for vaccine development because they provide targets for antibody that can block iron uptake and facilitate complement lysis and phagocytosis.

MATERIALS AND METHODS

General protocol. This vaccine efficacy study was a placebo-controlled, randomized and double blind study to demonstrate the effectiveness of an SE bacterial extract vaccine to protect chickens from SE colonization. Hens were vaccinated subcutaneously with 0.25 mL of vaccine in the back of the neck at 10 weeks of age and again eight weeks later. Control hens were similarly injected with a placebo vaccine containing adjuvant but without antigen. Hens were challenged four weeks later. Hens were swabbed in the cloaca twice weekly until day 13 of the challenge period, after which they were necropsied and their reproductive organs harvested. All samples were tested for the presence of the challenge organism by plating on brilliant-green nalidixic acid (NAL) agar. Data were analyzed by Preventive Fraction and Mitigated Fraction statistical methods (1).

Chickens. One hundred Specific Pathogen Free leghorn hens were obtained from Valo BioMedia, (Adel, IA) and grown to 10 weeks of age. The hens were tagged with numbered wing bands, randomly allocated into groups and commingled for the duration of the study.

Challenge. The challenge organism was a recent US field isolate and its identity was confirmed by the

Minnesota Poultry Testing Laboratory. The challenge organism was propagated in broth cultures and all hens were challenged with $\geq$ 1 x 10^8 colony-forming units (CFU).

RESULTS

Shedding of the challenge organism was monitored from cloacal swabs over 13 days after challenge. All hens were negative prior to challenge. On all days, the number of hens shedding was significantly reduced in vaccinates compared to placebo treated hens (Preventive Fraction analyses: Day 2 p=0.037, Day 6 p=0.002, Day 9 p=0.007 and Day 13 p=0.016). In addition, the severity of colonization of placebo treated hens was compared to vaccinates by Mitigated Fraction analyses. Consistent with the incidence data, the CFU values for the vaccinated group were significantly different than the placebo group on all four sample days after challenge.

On day 14 after challenge, all hens were necropsied and their reproductive organs were harvested and tested for the presence of SE. The placebo group had a reproductive organ colonization incidence of 24%, which was consistent with previously reported challenge studies with SE (2, 3) (Figure 1). No ovaries or oviducts from the vaccinated group were colonized with SE (Preventive Fraction analyses: 100% protection, p=0.016).

DISCUSSION

SE continues to be a problem in the poultry industry and the incidence is expected to increase as the use of antibiotics declines and as the movement to cage-free aviaries increases. Therefore, as part of effective control programs, the use of vaccines will intensify and it is important to provide the industry with current technologies that will be effective in this changing environment. An SRP vaccine is such a technology, eliciting immunity directly focused on a main pathogenic mechanism of SE; that is, its ability to acquire iron, and eliminating much of the irrelevant immunity and reactivity elicited by bacterins.

The SRP technology has been used in several commercial poultry operations for over 20 years in autogenous form to successfully control *Salmonella*, *E. coli* and fowl cholera. It is also used in the licensed bovine *Salmonella* Newport (4) and E. coli (5) vaccines where a reduction of shedding, reduced disease and improved herd performance have been reported. Similarly, Epitopix has gained USDA approval of efficacy for a Klebsiella mastitis vaccine (6) shown to reduce infection and to improve herd performance as well as a cross-protective *Pasteurella multocida* vaccine for chickens (reported in these proceedings).

In this study, the SE SRP vaccine was demonstrated to reduce cloacal shedding and prevent colonization of the reproductive organs. Reducing shedding is critical to prevent spread of *Salmonella* by the fecal-oral route in hens and the subsequent contamination of eggs and poultry for human consumption. Specific prevention of ovary and oviduct colonization is critical to prevent vertical transmission and contamination of eggs for consumption. Therefore, the ability of the SRP vaccine to effectively reduce shedding and prevent reproductive organ colonization confirms the utility of this vaccine in *Salmonella* control programs.

REFERENCES

Siev, D. An estimator of intervention effect on disease severity. J Modern Applied Statistical Methods, 4:500-508. 2005.

Gast, Richard K. and C.W. Beard. Isolation of Salmonella enteritidis from Internal Organs of Experimentally Infected Hens. Avian Diseases 34:991-993. 1990.

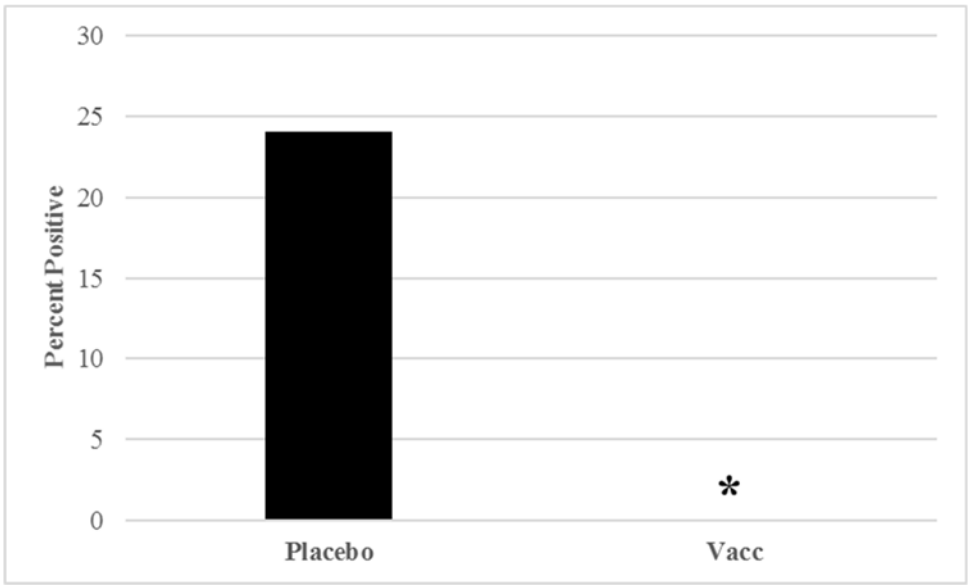
Gast, Richard K., Stone, Henry D., Holt, Peter S. and C.W. Beard. Evaluation of the Efficacy of an Oil-Emulsion Bacterin for Protecting Chickens against Salmonella Enteritidis. Avian Diseases 36: 992-999. 1992.

Hermesch, D.R. *et al.* Effects of commercially available vaccine against Salmonella enterica serotype Newport on milk production, somatic cell count, and shedding of Salmonella organisms with no clinical signs of salmonellosis. Am J Vet Res. 69: 1229-34. 2008.

Thomson, D.U., *et al.* Use of a siderophore receptor and porin proteins-based vaccine to control the burden of Escherichia coli O157 in feedlot cattle. Foodborne Pathog Dis. 6: 871-7. 2009.

Gorden, P.J., Kleinhenz, M.K., Ydstie, J.A., Slinden, L. and Burkhardt, D. Evaluation of a Novel Vaccine Based on Siderophore Receptor Proteins and Porins (SRP ® Technology) for Controlling Klebsiella Mastitis in a Dairy Herd. American Association of Bovine Practitioners conference proceedings (submitted for publication). 2016.

Figure 1. Prevention of SE Colonization of the Reproductive Organs by Vaccination. Percentage of hens positive for colonization of the reproductive organs with SE 14 days after challenge administration. * Indicates significant difference of vaccinates from the placebo group.



PET POULTRY COURSE FOR VETERINARIANS

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SUMMARY

Poultry (chickens and turkeys) are classified as a major food animal (1). The diagnosis of foreign animal diseases, such as highly pathogenic avian influenza (HPAI) and exotic Newcastle disease, have a major impact on the entire US poultry industry. With the recent outbreaks of HPAI in the USA and the world (2), it is essential that private veterinarians know how to recognize exotic diseases of poultry, collect proper samples for detection of these critical diseases and can provide advice on biosecurity and disease prevention for bird owners.

Private veterinarians have been presented with an increasing number of pet poultry in their clinics necessitating a demand for continuing education in poultry medicine, diagnosis, management and treatment. Following this continued demand from private veterinarians, a hands-on training course for non-poultry veterinarians was designed to demonstrate technical skills in poultry medicine, diseases and diagnostics. Participants were instructed on reportable diseases and how to collect proper samples for lab submission for a prompt and accurate diagnosis. The one day course was divided in to:

Lectures

Housing and behavior

Regulatory medicine, reportable diseases (i.e. avian influenza, Newcastle disease, infectious laryngotracheitis), import/export regulations, quarantine/hold orders and the FDA veterinary feed directive

Respiratory and reproductive conditions

Trauma, including pecking and pododermatitis

Marek's disease and vaccination

External and internal parasites, management and treatment

Wet lab

History taking, physical examination, sample collection

Anesthesia, intubation, X-ray

Euthanasia techniques

The course was received with enthusiasm by practitioners. Self-rated knowledge and abilities changed for the majority of participants at the conclusion of the course. Participants found the topics on vaccination, sample collection, anesthesia, and euthanasia most helpful. Participants requested additional hands-on continuing education training on poultry. They asked that future courses provide more specifics on medications. Some participants indicated that providing a brief review on basic physiology and nutrition would be beneficial also.

Veterinarians are often on the frontline of public health issues. Training on poultry can help with the early detection of reportable diseases and diseases of economic importance. It is important for states' veterinary associations and Universities to encourage continuing education of poultry, through case studies and wet labs.

REFERENCES

1. Food and Drug Administration. Guidance for Industry #191. In: U.S. Department of Health and Human Services. 2015.

2. World Organisation for Animal Health (OIE). Avian Influenza Portal. 2017.http://www.oie.int/animal-health-in-the-world/web-portal-on-avian-influenza/: http://www.oie.int/animal-health-in-the-world/web-portal-on-avian-influenza/ Accessed on: Jan 16, 2017

TENOSYNOVITIS OUTBREAKS IN COMMERCIAL BROILERS ASSOCIATED WITH AVIAN REOVIRUS VARIANTS

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SUMMARY

During 2016 the California Animal Health and Food Safety Laboratory System (CAHFS), Turlock branch, received a total of 78 submissions of commercial broiler chickens with a history of splayed legs and poor growth performance. At necropsy, lesions suggestive of tenosynovitis and brittle bones were noticed. Microscopically lymphocytic and/or heterophilic tenosynovitis, lympho-follicular epicarditis and changes compatible with rickets were the most common findings. Reovirus was isolated from both tendon and heart tissue pools inoculated in chicken-embryo liver cells and confirmed by direct electron microscopy. These results, together with molecular characterization and full genome sequencing of a few selected isolates, supported the involvement of avian reovirus variants unrelated to the vaccine strain S1133. An overview of the outbreaks will be provided.

INTRODUCTION

Avian reoviruses (ARV) are non-enveloped double stranded RNA viruses with a segmented genome that can be both horizontally and vertically transmitted. They have been associated with a variety of conditions including viral arthritis/tenosynovitis, runting-stunting syndrome, brittle bones, hepatitis and immunosuppression. Viral arthritis/tenosynovitis represents an important disease in meat-type chickens (3). Economic losses result from poor growth performance and high condemnation rates at the processing plant. Control is typically achieved through vaccination of breeder flocks with live attenuated or inactivated products (3, 10). However, antigenically different viruses can overcome vaccine immunity. Recently, newly emerging ARV variants have been identified in United States (2, 5, 6, 7), Canada (1) and Europe (9). As segmented viruses, ARV genes can reassort (antigenic shift) to generate significant diversity in the genotype and pathotype, through a direct exchange of genome segments (4, 8). Point mutations (antigenic drift) play also an important role. The genetic diversity between these variants and classic vaccine strains has resulted in immunological escapes and vaccine failure in breeder flocks with routine ARV immunity programs.

MATERIALS AND METHODS

During 2016, the CAHFS, Turlock branch, received a total of 78 submissions of commercial broiler chickens with a history of splayed legs and poor growth performance. Live and dead chickens were sent for diagnostic evaluation. Necropsy was performed on all submissions and tissue sections were collected for histopathology and processed using hematoxylin and eosin staining. Joint and heart swabs were collected and tested for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by real time polymerase chain reaction (qPCR). Avian influenza real time reverse transcriptase (RRT)-PCR was performed on oropharyngeal swabs from all cases. Selected organs were cultured for aerobic bacteria using proper media (5% sheep's blood and MacConkey's agar). Virus isolation using avian cell culture inoculation and direct electron microscopy were performed on tendon tissue pools, heart tissue pools and synovial swabs obtained from necropsy cases (CAHFS, Davis). A few selected isolates were sent for molecular characterization, using reverse transcriptase (RT)-PCR targeting the Sigma C gene, and full genome sequencing (School of Veterinary Medicine, Davis). In addition, serotyping and biological characterization, through foot pad inoculation in one-day-old broiler chickens was performed at AviServe LLC. Blood samples were collected and sera were tested for infectious bronchitis, Newcastle disease, *Mycoplasma*

gallisepticum, Mycoplasma synoviae, avian influenza, avian reovirus and infectious bursal disease by enzyme-linked immunosorbent assay (ELISA).

RESULTS AND DISCUSSION

Submitted chickens ranged from 10 to 47 days of age. Birds between four and five weeks of age were most commonly affected (33%), followed by birds between two and three weeks of age (26%). Clinically, the birds exhibited difficulty standing and walking. Secondary traumatic lesions, including hematomas and wing and limb fractures, were frequently observed. At necropsy, lesions suggestive of tenosynovitis, femoral head necrosis and brittle bones were noticed. Septicemic lesions were found in 18% of cases (n=14). In these cases *Escherichia coli* (n=10), followed by *Enterococcus* spp. (n=3) and *Staphyloccus aureus* (n=1) were isolated from several organs by aerobic cultures. These bacteria may have represented secondary opportunistic agents. Joint and heart swabs for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* were negative by qPCR. Microscopically, lymphocytic and/or heterophilic tenosynovitis, lympho-follicular epicarditis and changes compatible with rickets were the most common findings. Reovirus was isolated from tendon/synovial fluid (53 positive/ 74 tested) and heart (10 positive/29 tested) in chicken-embryo liver cells and confirmed by direct electron microscopy. These findings supported the viral etiology of the outbreaks and suggested the role of reovirus as a primary pathogen. Interestingly, lesions indicative of rickets were noticed in a majority of cases. Typically, rickets occurs in young birds as a result of nutritional deficiencies, including lack of vitamin D, calcium, phosphorus and an imbalanced ratio of these two minerals. Reovirus has also been associated with skeletal disorders such as brittle bones and osteoporosis (3). Molecular characterization and genome sequencing determined that reovirus isolates obtained from clinical cases were unrelated to the vaccine stain S1133 (detailed results provided by Dr. Gallardo et al. In 66th Western Poultry Disease Conference, Sacramento, CA, 2017). These findings were compatible with those reported by Dr. H. Sellers (University of Georgia) in which ARV variants circulating in broiler flocks were in some cases less than 50% homologous to commercial vaccine strains (6,7). Three of the isolates sent for biological characterization appeared to be highly pathogenic, producing severe foot pad and systemic lesions, including hydropericardium, hepatic necrosis, and thin - walled small intestine and stunting. It was not possible to identify a distinct serotype, however these results suggest the existence of reovirus variants. Sera collected from birds between five and six weeks of age frequently showed positive titers for reovirus (titer groups 7-9). In summary these results support the involvement of avian reovirus variants in the submissions received at CAHFS, Turlock branch, during 2016.

ACKNOWLEDGMENT

Reovirus serotyping and biological characterization was done by Dr. Rosenberger at AviServe LLC, Delaware Technology Park, Newark.

REFERENCES

Ayalew, L., A. Gupta, J. Fricke, B. Lockerbie, S. Popowich, and S. Gomis. Isolation and characterization of avian reoviruses by phylogenetic analysis and restriction enzyme fragment length polymorphism (RFLP). In: 65th Western Poultry Disease Conference, Vancouver, Canada, 2016.

Dale, E., H. Sellers, and E. Linnemann. Pathogenesis of two variant reoviruses from clinical cases of tenosynovitis. In: 63th Western Poultry Disease Conference, Puerto Vallarta, Mexico. 2014.

Jones, C.R. Reovirus infections. In: Diseases of Poultry, 13th ed. D. E. Swayne. John Wiley & Sons, Inc. pp 351-372. 2013.

Kawamura, H., F. Shimizu, M. Maeda, and H. Tsubahara. Avian reovirus: Its properties and serological classification. Natn Inst Anim Hlth Qt, Tokyo. 5:115-124. 1965.

Lu, H. , Y. Tang, P. A. Dunn, E. A. Wallner - Pendleton, L. Lin, and E. A. Knoll. Isolation and molecular characterization of newly emerging avian reovirus variants and novel strains in Pennsylvania, USA, 2011-2014. Sci Rep. 15; 5: 14727. 2015.

Sellers, H., V. Durairaj, V. Gauthiersloan, and E. Linnemann. Genotypic analysis of avian reovirus from clinical submissions of viral arthritis. In: 64th Western Poultry Disease Conference, Sacramento, CA. 2015.

Seller, H., E. Linnemann, V. Gauthiersloan, and T. Collett. Isolation and characterization of reovirus field isolates from clinical cases of viral arthritis. In: 62th Western Poultry Disease Conference, Sacramento, CA. 2013.

Su, Y. P., B. S. Su, J. H. Shien, H. J. Liu, and L. H. Lee. The sequence and phylogenetic analysis of avian reovirus genome segments M1, M2, and M3 encoding the minor core protein muA, the major outer capsid protein muB, and the nonstructural protein muNS. J Virol Methods. 133:146-157. 2006.

Troxler, S., P. Rigomier, I. Bilic, D. Liebhart, I. Prokofieva, and B. Robineau, M. Hess. Identification of a new reovirus causing substantial losses in broiler

production in France, despite routine vaccination of breeders. Vet Rec. 25; 172 (21):556. 2013.

Van der Heide, L. The History of Avian Reovirus. Avian. Dis. 44:638-641. 2000.

SALT TOXICITY IN SEVEN-DAY-OLD BROILER CHICKENS

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INTRODUCTION

Salt poisoning in commercial poultry can result in severe economic losses. This condition is associated with excessive ingestion of salt (sodium chloride), following the consumption of either saline water or, more commonly, an improperly formulated feed (7). Toxicity is related to the sodium ion (Na+). Young birds appear more susceptible due to their immature renal function (9). Clinical signs include diarrhea, dehydration, weakness and neurological disorders (3). Changes indicative of heart failure are frequently observed. Brain lesions have also been reported (2). This report presents a case of severe cardiovascular changes and cystic degeneration of testes in seven - day-old commercial broiler chickens associated with consumption of excessive dietary salt. The history included high mortality (1-2% per day) associated with anasarca, respiratory distress and diarrhea in a flock of 28000 birds. Immediate substitution of the imbalanced feed resulted in resolution of clinical signs and return to normal mortality within two days.

RESULTS AND DISCUSSION

Eighteen live and two dead, seven-day-old, broiler chickens were submitted to the California Animal Health and Food Safety Laboratory System (CAHFS), Turlock branch, for necropsy. Severe anasarca and changes indicative of heart failure, including hydropericardium, ascites, pulmonary congestion and dark red mottled livers, were the most commonly observed gross findings. The reproductive tract of a few birds showed cystic degeneration of testes. In addition, multifocal hemorrhages, visible from the serosal surface of ceca, were noticed in one bird. Toxicology screening of feed for salt content revealed high levels of Na+ (1.2%), exceeding six times the recommended levels for poultry (0.2%) (4). High levels of potassium (1%), exceeding three times reference values (0.3%) were also detected. Tissue sections were processed for light histopathology using hematoxylin and eosin staining. Histopathology showed lesions indicative of heart failure and hypoxia, further confirming the clinical pathology and toxicology results. Brain lesions, frequently reported in case of sodium poisoning, were mild in this case. This finding suggest an acute exposure.

Once absorbed, Na+ induces water retention with hypervolemia and reduced blood cell deformability, leading to right heart failure and ascites (3, 8). Cystic degeneration of testes is typically associated with salt poisoning. Possible explanations for this condition include fluid retention due to an osmotic effect or abnormal fluid secretion by seminiferous tubules. High Na+ levels might stimulate the secretory activity of seminiferous tubules. Since in young birds the efferent ducts are still patent, continuous secretion of fluids against pressure can lead to cyst formation and compression of the epithelium (6). Gastroenteritis and intestinal hemorrhages have been described in cases of salt toxicity, due to the irritant effect of Na+ (5). In this case hemorrhages were localized to the cecal mucosa of one bird. Ceca are actively involved in water reabsorption, leading to Na+ concentration at this level and a prolonged contact time with the mucosa. Hypoxia due to heart failure might also have played a role. Potassium is involved in the maintenance of membrane potential and cellular fluid balance. It is also essential for normal heart activity (4). Hyperkalemia has been associated with electrocardiographic changes (elevation of the T-waves), sinus bradycardia and sinus arrests in ducks (1). A similar mechanism cannot be excluded in this case, with a cumulative effect on an already compromised heart function.

REFERENCES

Andersen, H. T. Hyperpotassemia and electrocardiographic changes in the duck during prolonged diving. Acta Physiol. Scand. 63:292-5. 1965.

Crespo, R., M. Subbiah, C. Corsiglia, A. Bickford, and B. Puschner. Bilateral Malacia Associated with Sodium Poisoning in Turkey Poults. Avian Dis. 52:179–182. 2008.

Fulton, R. M. Toxins and poisons. In: Diseases of Poultry, 13th ed. D. E. Swayne. John Wiley & Sons, Inc. pp 1293-1294. 2013.

Klasing, K. C. Nutritional diseases. In: Diseases of Poultry, 13th ed. D. E. Swayne. John Wiley & Sons Inc. pp 1205-1232. 2013.

Osweiler, G. D., T. L. Carson, W.B. Buck, and G. A. Van G-elder. A. Water deprivation-Sodium Salt. In: Clinical and diagnostic veterinary toxicology. 3rd. ed. Kendall Hunt Publishing Company, Dubuque, IA. 167-170. 1985.

Siller, W. G., W. A. Dewar, and C.C. Whitehead. Cystic dilatation of the seminiferous tubules in the fowl: a sequel of sodium intoxication. J. Pathol. 107(3):191-7. 1972.

Siller, W. G., Renal Pathology of the fowl: A review. Avian Pathol. 10: 187-262. 1981.

Thompson, L. J. Sodium Chloride (Salt). In Veterinary Toxicology: Basic and Clinical Principles. 2nd ed. R. C. Gupta, Elsevier. pp. 559-560. 2012.

Wages, D. P., M. D. Ficken, M. E. Cook, and J. Mitchel. Salt toxicity in commercial turkeys. Avian Dis. 39: 158-161. 1995.

CALIFORNIA BACKYARD POULTRY AS A RESERVOIR FOR RESPIRATORY DISEASES

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INTRODUCTION

Raising backyard chickens is an ever growing hobby in the United States. Within the next five years 4% of the population of some of the largest cities in the United States plan on getting chickens. Currently, almost 1% of the population in the same cities already own chickens (9). Despite the growing interest in backyard poultry it has been very little research conducted on backyard poultry flocks. Even though, available studies have mostly involved mailing surveys to owners and poultry disease screening (5, 6, 7), this is the first study looking at the epidemiology of respiratory diseases and backyard chickens and its association to commercial poultry production. Respiratory diseases represent a significant proportion of diagnosed diseases from backyard poultry flock laboratory submissions (8). In commercial settings respiratory diseases are extremely important particularly because of the losses they generate. The latest Exotic Newcastle Disease outbreak of 2002 in California was associated with backyard poultry tenancy. In addition, Avian Influenza outbreaks have also been associated to backyard flock presence. As the number of backyard chickens increases in our state it can be suspected potential for more disease amplification and transmission to commercial facilities.

MATERIALS AND METHODS

Biosecurity survey. A survey was created to assess the management and biosecurity of backyard chicken flocks. The survey was modeled after a biosecurity assessment of commercial poultry facilities (4). The survey consists of 34 questions and was conducted face-to-face with the participants at their property. The same interviewer was used every time the survey was conducted.

Backyard flocks. All animal experimental procedures were approved by the University of California- Davis Institute Animal Care and Use Committee. Backyard poultry flocks were located and contacted using the California Backyard Poultry Census. Backyard poultry flocks were considered "close" to commercial poultry facilities if they were located within four miles of a known large scale commercial poultry producer, premises more than four miles from commercial poultry were considered "far" from commercial poultry. A total of 41 backyard flocks were used totaling 584 birds. Twenty-seven flocks totaling 329 birds were considered far from commercial poultry facilities and 14 flocks totaling 255 birds were considered close to commercial poultry facilities. The size of the backyard flocks used ranged from 2-400 birds and all flocks were located in Yolo, Sonoma, Napa, Alameda, and Yuba counties in California. All of the flocks were visited between January and September of 2016.

Serological Tests. Antibodies against *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Ornithobacterium rhinotracheale* (ORT), Infectious Bronchitis (IBV), Newcastle Disease (NDV), Infectious Laryngotracheitis (ILT), and avian influenza (AI) were assessed using commercial ELISA kits using a spectrophotometer.

Statistical analysis was performed using a chi-square test, statistical significance was determined at $p<0.05$.

RESULTS

Biosecurity survey. The survey responses show that many backyard poultry owners do not implement bio-security practices which could reduce disease risk in their flocks. The bio-security measures that are most commonly lacking are: having dedicated chicken shoes, obtaining chickens from NPIP certified sources, using the California Animal Health and Food Safety Laboratory service to diagnose mortality, keeping visitors away from the flock, and keeping wild birds from intermingling with the flock.

Serology. The serology results show that antibodies against all tested diseases were found in backyard flocks in California. Results show that ORT, IBV, MS, MG, and ILT are prevalent in backyard

poultry flocks with 97%, 75%, 73%, 70%, and 49%, respectively, of flocks testing positive for antibodies against each disease. Five flocks did test positive for avian influenza with an ELISA kit but subsequent PCR tests from oropharyngeal swabs were negative for the virus. There was no significant difference for distance to the nearest commercial poultry facility and increased risk of each respiratory disease.

DISCUSSION

Backyard poultry owners showed to be lackadaisical when it comes to implementing simple biosecurity measures such as, using dedicated shoes or clothes to work with poultry. One of the easiest measures to avoid food safety issues when working with livestock is using dedicated clothes and shoes. In addition, hand washing / sanitizing plays a huge role in controlling zoonoses (1). Backyard poultry owners need to be educated and surveillance needs to be performed in order to constantly evaluate the risks posed to the commercial poultry production. More than 70% of the surveyed premises get their chickens from non-regulated premises. This is particularly important to control vertically transmitted diseases e.g. *Salmonella* and *mycoplasmas.* Only 35% of the flock owners consult a lab or veterinarian regarding health issues. This is an important topic that needs to be addressed in training sessions to backyard flock owners.

Results show that ORT, IBV, MS, MG, and ILT are prevalent in backyard poultry flocks, as shown by a prior study conducted in Belgium (2). Seroprevalence values for NDV, ILT, and MG differ from a study in Maryland where high seroprevalence to ILT low to MG and NDV were detected (5). The differences in the antibody profile may indicate different risks for different respiratory diseases depending on the geographic location. It should be noted that one premise was vaccinated against NDV and IBV, which gave the positive results for that flock which was far from a commercial facility. While no significant difference for distance to the nearest commercial poultry facility and increased risk of each respiratory disease was found in the present study, it should be noted that those people who live near commercial facilities were more difficult to contact and less likely to participate than other backyard poultry owners. This study does show that due to the antibody profiles found in backyard poultry flocks that backyard poultry flocks have the potential to serve as a reservoir for respiratory diseases.

REFERENCES

1. Grunkemeyer, V. L. Zoonoses, Pulic Health, and the Backyard Poultry Flock. Veterinary Clinics of North America: Exotic Animal Practice. 14(3): 477-490. 2011

2. Haesendonck, R, Verlinden, M., Devos, G., Michiels, T., Butaye, P., Haesbrouk, F., Pasmans, F., and Martel, A.

3. High Seroprevalence of Respiratory Pathogens in Hobby Poultry. Avian Diseases, 58(4): 623-627. 2014

4. Hauck, M. R., Crossley, B., Rejmanek, D., Zhou, H., and Gallardo, R. A. Persistence of High and Low Pathogenic Avian Influenza Viruses in Footbaths and Poultry Manure. Avian Diseases In-Press. 2016

5. Madsen, J. M., Zimmermann, N. G., Timmons, J., and Tablante, N. L. Avian Influenza Seroprevalence and Biosecurity Risk Factors in Maryland Backyard Poultry: A Cross-Sectional Study. PLoS ONE, 8(2): e56851. 2013

6. McBride, M. D., Hird, D. W., Carpenter, T. E., Snipes, K. P., Danaye-Elmi, C., Utterback, W. W. Health Survey of Backyard Poultry and Other Avian Species Located Within One Mile of Commercial California Meat-Turkey Flocks. Avian Diseases, 35(2): 403-407. 1991

7. Smith, G. and Dunipace, S. How Backyard Poultry Flocks Influence the Effort Required to Curtail Avian Influenza Epidemics in Commercial Poultry Flocks. Epidemics, 3:71-75. 2011

8. Stinson, S. and Mete, A. Popular Backyard Flock Program Reduces Biosecurity Risks of Amateur Production. California Agriculture, 67:23-209. 2013

9. USDA-NARMS. Urban Chicken Ownership in Four U.S. Cities. Poultry 2010. 2012

DETECTION OF NEW INFECTIOUS BRONCHITIS VIRUS (IBV) VARIANTS IN THE US AND EUROPE USING PROFLOK® IBV AB ELISA

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ABSTRACT

Infectious bronchitis (IB) continues to be an important disease in commercial poultry worldwide (1). With the propensity of Infectious bronchitis virus (IBV) for antigenic mutations, quick detection of new variant IBV is critical in controlling the disease. ProFLOK® IBV Ab ELISA was developed to detect field infection even in hyperimmunized flocks. These new studies were selected to demonstrate the ability of ProFLOK IBV to detect antibodies to various IBV serotypes commonly observed in the US and Europe. Serum samples from commercial and experimental flocks exposed to various field and/or vaccine IBV serotypes were tested. These studies show that ProFLOK IBV Ab ELISA kits are able to detect antibodies to common and new IBV variants.

INTRODUCTION

IB is a highly contagious, acute, upper respiratory tract infection in chickens caused by an avian coronavirus IBV (1). The disease has worldwide distribution and is of high economic importance to the poultry industry. Clinical signs may be mild but the infection may result in egg production problems and predispose the birds to secondary infections and cause significant effects on flock health and performance.

IBV is an enveloped virus with single-stranded RNA that codes for several important structural genes including the spike gene, S1, which is a critical serotype determinant. There are a number of types, subtypes, and variants of IBV that have been isolated and described due to its high mutation rate and recombination events. Viral replication often occurs with high error rates due to limited proof-reading capabilities of its RNA polymerase resulting in substitutions, insertions and deletions in the viral genome. Mutations and/or recombinations in the genome produce new strains or serotypes of the virus in the field, making control of IBV infections extremely challenging (1).

ProFLOK IBV Ab ELISA (ProFLOK IBV, Zoetis Inc.) is an indirect, antibody ELISA test kit used to detect antibodies to IBV in chicken sera (2). The kit was developed at a time when there was high respiratory challenge in the Delmarva region and it was specifically designed to have increased sensitivity to detect IBV infection even in hyperimmunized flocks. The increased sensitivity results in higher antibody titers compared to other commercially available antibody ELISA kits (3). The antibody titer difference leads to questions about its performance and its ability to detect new and emerging variant IBV in the field.

We present several studies conducted in the US and in Europe to show evidence of the performance and the utility of ProFLOK IBV in monitoring immune response to IBV vaccination or infection and its ability in detecting antibodies to new variants of IBV. These studies varied from controlled vaccine-protection ("protectotype") studies or field vaccine studies.

STUDY RESULTS

The first protectotype study (4) was conducted in commercial broilers where different IBV vaccination programs were evaluated to determine protection from various field challenges. All birds were vaccinated with a live Massachusetts (MASS) type IBV vaccine administered intraocularly on day one of age and were given a booster vaccine on day 14 with either the same MASS vaccine, a mid-passage Massachusetts (MASSII) vaccine, or a combination of Georgia 98 and Arkansas (GA98+ARK) vaccines. Each vaccination group was subdivided into four virus challenge groups which were challenged on day 28 with either Massachusetts 41 (MASS41), Arkansas DPI (ARK), Delaware 072 (DE/072/92), or Georgia 08 (GA08). Vaccinated controls, challenge controls and negative controls were also included in the study. Measurement of weight gain, level of airsacculitis, ciliostasis of tracheal cilia, and tissue samples for histopathology and virus isolation were taken five days post-challenge (DPC) to evaluate the level of protection provided by the various vaccine programs. Serum samples were taken at five DPC and tested with

ProFLOK IBV and a competitor IBV antibody ELISA (Kit B) to determine the immune response after challenge. The serology results showed that while there were no significant differences in mean antibody titers between the vaccination program groups, there was a trend for higher antibody titers as measured by ProFLOK IBV compared to Kit B. In addition, the ProFLOK IBV antibody titers showed more visible increases in antibody levels in the groups that were challenged with heterologous IBV serotypes, especially showing high maximum antibody titers in the DE/072/92 and GA08 challenged groups.

In another protectotype study (5), commercial broilers were vaccinated with either a commercially-prepared Georgia 08 (ZTS GA08) or an autogenous Georgia 08 (Auto GA08) vaccine. Each vaccine group was divided further into a single-dose vaccine group where the GA08 vaccine was administered only on day one or a two-dose vaccine group where the GA08 vaccines were administered on day one and day 14. Each vaccine group was either challenged with a Zoetis GA08 isolate or with an autogenous GA08 field isolate on day 35. Non-vaccinated controls for each challenge virus were also included in the study. Serum samples were taken on six DPC and tested with either ProFLOK IBV or another commercial IBV Ab ELISA (Kit B). The results of the serology showed that ProFLOK IBV is able to detect immune response to GA08 vaccination and challenge and tend to have higher geometric mean antibody titers compared to Kit B just 5 days post-challenge.

The third study (6) was conducted in Europe to demonstrate the ability of ProFLOK IBV in detecting antibodies to the variant QX virus. Eleven broiler flocks in the Netherlands were vaccinated with a combination of Holland 120 (H120) and D274 IBV on day one via coarse spray. A second vaccination using variant QX vaccine was administered via coarse spray or drinking water around days 10-14. Serum samples were taken at various times ranging from six to 27 days post-second vaccination and tested using ProFLOK IBV and two other commercial IBV ELISA kits (Kit B, Kit C). Serology showed that all ELISA kits were able to detect the immune response to QX vaccination around three weeks post-vaccination with ProFLOK IBV showing higher antibody titers compared to the other commercial ELISA kits.

DISCUSSION/CONCLUSIONS

These studies demonstrate that ProFLOK IBV Ab ELISA kits are able to detect immune response to various serotypes of IBV vaccines or challenge. ProFLOK IBV can detect antibodies not only to the common vaccine serotypes but also to IBV variants GA08 in the US and QX in Europe.

These studies also show the utility of using ProFLOK IBV in monitoring immune response even in hyperimmunized flocks. This feature of ProFLOK IBV detecting immune response as early as five to six DPC and high antibody titers especially in the presence of heterologous virus challenge can provide an early indication of field exposure and help in flock health management and IBV vaccination programs.

REFERENCES

1. Jackwood, M. W., and S. de Wit. Infectious Bronchitis. In: Diseases of Poultry, 13th ed. D. E. Swayne, ed. John Wiley & Sons, Inc., Iowa. pp 139-159. 2013.

2. ProFLOK Infectious Bronchitis Virus Antibody Test Kit Direction Insert, USDA Product Code 5030.00, Zoetis Inc.

3. Study Report No. 02-12-70AQO, Zoetis Inc.

4. Study Report No. 15-13-70AQO, Zoetis Inc.

5. Study Report No. 28-14-70AQO, Zoetis Inc.

6. Study Report No. D819R-FR-16-028, Zoetis Inc.

Table 1. Infectious bronchitis virus antibody ELISA titers measured at 5 days post-challenge using ProFLOK® IBV Ab ELISA and Kit B.

Virus Challenge	ProFLOK® IBV			Kit B		
ARK DPI	Geometric Mean Titer	Lower Limit	Upper Limit	Geometric Mean Titer	Lower Limit	Upper Limit
MASS/MASS	1705.84	991.97	2932.94	540.75	335.30	871.62
MASS/GA98+ARK	1147.60	88.78	14693.08	410.13	57.20	2902.87
MASS/MASSII	3879.43	1915.39	7856.31	621.29	345.92	1115.10
Non-vaccinated	3.47			12.27		
DE/072/92						
MASS/MASS	1993.40	536.54	7398.74	466.45	179.60	1209.03
MASS/GA98+ARK	2151.10	212.02	21740.97	639.72	83.30	4869.25
MASS/MASSII	507.98	72.85	3506.64	348.04	178.11	679.28
Non-vaccinated	1.94			5.45		
GA08						
MASS/MASS	1552.24	431.03	5583.78	568.81	228.77	1411.90
MASS/GA98+ARK	7200.18	3609.13	14364.72	1551.93	830.39	2899.97
MASS/MASSII	5003.04	1818.47	13762.84	704.50	379.43	1307.19
Non-vaccinated	0.00			3.24		
MASS41						
MASS/MASS	735.57	58.39	9133.37	454.27	128.74	1596.67
MASS/GA98+ARK	6480.45	2812.54	14930.08	1604.03	919.30	2798.23
MASS/MASSII	380.65	22.82	6114.61	388.32	129.23	1162.86
Non-vaccinated	2.40			49.10		
Non-Challenged						
MASS/MASS	41.63			111.98		
MASS/GA98+ARK	1718.86			448.39		
MASS/MASSII	287.99			161.16		
Non-vaccinated	0.00			0.76		

No significant differences among vaccinated challenged groups

MOLECULAR EPIDEMIOLOGY OF REOVIRUSES IN CALIFORNIA

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INTRODUCTION

During the last two years the California Animal Health and Food Safety Laboratory (CAHFS) has received numerous submissions from meat type chickens with a history of leg problems (splayed legs, valgus and brittle bones), poor performances (stunting and feed conversion) and lack of uniformity. From these submissions 122 reoviruses have been isolated and confirmed by electron microscopy (EM). The aim of this investigation was to do a molecular biological characterization of isolates that were associated with lesions and clinical signs comparing them with related isolates.

MATERIALS AND METHODS

Eight reovirus isolates were received for molecular characterization. An RT-PCR focusing on the Sigma C gene of the reovirus was performed (Kant et al., 2003). The generated amplicons (1088bp) were sequenced in order to create a phylogenetic tree for comparison. Additionally, all the received isolates were used to prepare cDNA libraries and these libraries were sequenced using an Illumina HiSeq 3000.

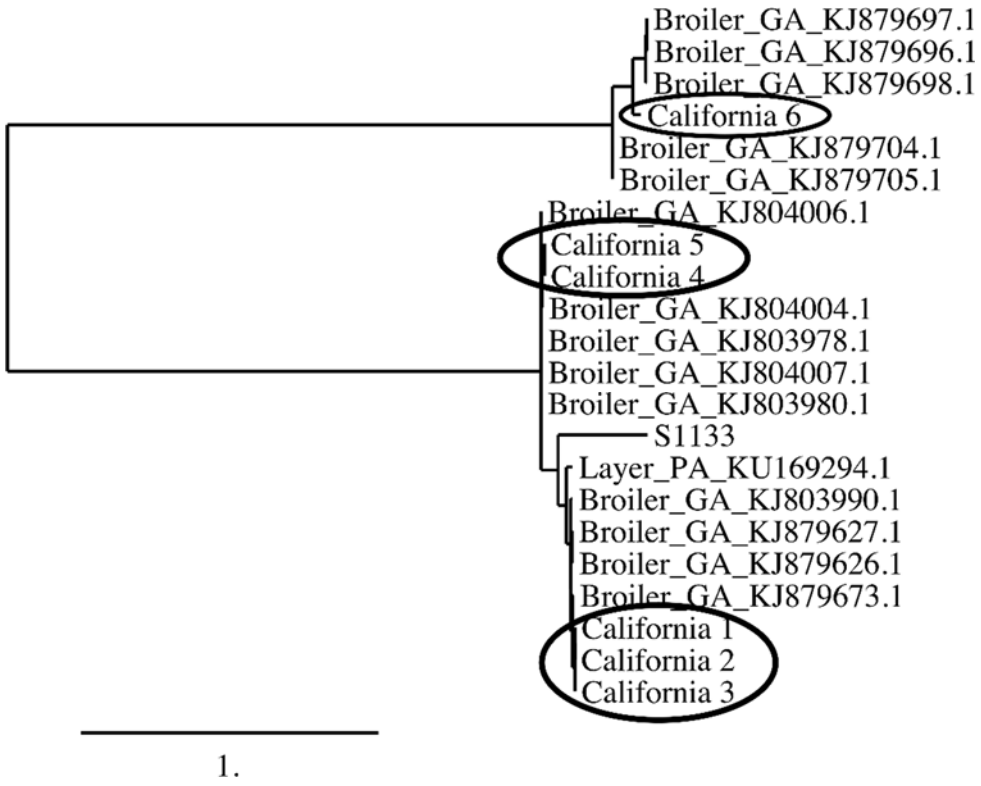
In a second stage 20 additional reovirus isolates were chosen from a total of 122 considering their cytopathic effect (CPE) in cells and the tissues from where they were isolated. Currently these isolates are characterized by phylogenetic analysis of the same PCR product.

RESULTS AND DISCUSSION

From the first batch of eight isolates only six viruses were effectively amplified by conventional RT-PCR. The phylogenetic tree in Fig. 1 shows the molecular distance between these viruses and to the vaccine virus S1133 (Fig. 1). Three major clusters can be seen in the tree. S1133 does not seem to be part of any cluster being 53% homologous to California 6 and 78% homologous to the rest of the isolates. The homology between the two lower clusters is 90%. The homology between the two lower clusters and the above cluster is 50%. These results suggest the presence of variants circulating in the commercial broiler population in California. All California isolates were most closely related to reovirus isolates from broilers in Georgia.

In order to better understand the circulating reoviruses the eight isolates were submitted for full genome sequencing. Preliminary results suggest that S1 is the gene that differs more on these viruses followed by L3 and M2. We are in the process of further analyzing the genomes and obtaining the sequences of the second batch of isolates.

Figure 1. Phylogenetic tree of the first 6 isolates in which their Sigma C gene was sequenced including vaccine strain S1133 as well as the most similar sequences from Genbank.



IMMUNOPROTECTIVE EFFECTS OF CPG-ODN AGAINST *E. COLI* SEPTICEMIA IN NEONATAL BROILER CHICKENS BY INTRAPULMONARY (IPL) DELIVERY

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SUMMARY

Immune stimulatory properties of Cytosine-Phosphodiester-Guanine oligonucleotides (CpG-ODNs) against bacterial infections in neonatal broiler chickens have been demonstrated previously. *In-ovo* delivery of CpG-ODNs was able to protect neonatal broiler chicks against bacterial infections such as *E. coli* and *Salmonella typhimurium* at a significant level. The following experiment was conducted to explore the effect of CpG-ODNs by intrapulmonary delivery (IPL) in neonatal broiler chicks. Day old broiler chicks (n=40) were exposed to IPL delivery of CpG-ODN microdroplets. Another group of chicks received normal saline in the same delivery method. At day three post-hatch chicks were challenged with a lethal dose of *E. coli* and they were monitored for mortality and clinical signs for 10 days following challenge. A daily clinical score was assigned to each bird and a cumulative clinical score (CCS) was calculated at the end of the experiment. Bacterial swabs were taken from air sacs from dead or euthanized birds and cultured on blood agar to enumerate bacterial load. The group that received IPL CpG-ODN was significantly protected against *E. coli* challenge compared to the control group that received saline ($P < 0.05$). Birds that received IPL CpG-ODN had also lower clinical score compared to the control group received saline ($P < 0.05$). This study has demonstrated utility of CpG-ODN as an immunostimulant against *E. coli* infections in neonatal boiler chickens by IPL delivery.

INTRODUCTION

High mortality associated with bacterial infections during the first week of a birds life can cause significant economic losses to the poultry producer(14). Among neonatal inactions, *Escherichia coli* infections are the most common and leading to increased first week mortality, lack of flock uniformity, chronic infections and increased condemnations at processing (3, 9). In order to prevent these economic implications in the poultry industry, prophylactic use of antibiotics have been in practice commonly. However, these industry practices have led to the emergence of resistant strains of bacteria and antibiotic residues in poultry products (4, 8). As a result, poultry industry is in search of alternatives to antibiotics and potential immunotherapeutic agents. Our group has identified CpG-ODNs as a stimulant of the innate immune system of chickens that protects them against lethal *E. coli* septicemia and *Salmonella typhimurium* infections by *in-ovo* delivery in embryonating eggs at day 18 of age (6, 7, 12). Furthermore, we have demonstrated that immunoprotective effects of CpG-ODN against septicemia in chickens were due to a Th1 type immune mechanism (11). The objective of this study was to identify immunoprotective effects of CpG-ODN by the intrapulmonary delivery as micro-droplets in neonatal broiler chickens at the time hatch.

MATERIALS AND METHOD

CpG-ODN and intrapulmonary delivery. The CpG-ODN (TCGTCGTTGTCGTTTTGTCGTT [(2007)]) was free of endotoxin and produced with a phosphorothioate backbone (Operon Biotechnologies, Inc. Huntsville, AL). Synthetic CpG-ODN was diluted in sterile, non-pyrogenic saline. IPL CpG-ODN was aerosolized to a group one day old broiler chicks (n=40) as micro-droplets using a *Compressor Nebulizer (705-470)* unit (AMG Medical Inc., Montreal, QC, H4T 1V5) at a concentration of 111mg/m^3 in closed 0.036 m^3 acrylic chamber for 30 minutes. The control group of birds (n=40) was aerosolized with saline for 30 min in the acrylic

chamber using the Compressor Nebulizer. The temperature was maintained at 28-30 C in the acrylic chamber during administration of CpG-ODN or saline.

***E. coli* culture, animal model, and experimental design**. A field isolate of *E. coli* from a turkey with septicemia was used as the challenge strain as previously described (7, 13). The *E. coli* animal challenge studies were conducted as described previously (6). Briefly, birds were challenged with either $1x10^4$ (n=20) or $1x10^5$ cfu of *E. coli* (n=20) by the subcutaneous route in the neck at 3 days post hatch (2 days following IPL micro-droplet delivery). Two doses of *E. coli* were given to each group of birds to simulate field conditions since all birds in a commercial poultry barn will not be exposed to a consistent dose of *E. coli*. Birds were evaluated three times daily at the critical stage (until three days post-challenge) and twice thereafter for 10 days post-challenge. Each bird was observed for clinical signs and a daily clinical score was assigned: 0 = normal; 0.5 = slightly abnormal appearance, slow to move; 1= depressed, reluctant to move; 1.5 = reluctant to move, may take a drink and peck some; 2 = unable to stand or reach for food or water; and 3 = found dead. Birds that received a clinical score of two were euthanized by cervical dislocation. At the end of the trial, each bird was given a cumulative clinical score (CCS) as a sum of daily clinical scores as previously described (7).

Chicks that were found dead or euthanized were necropsied immediately. On day seven post-challenge, the remaining birds were euthanized by cervical dislocation. Bacterial swabs were taken from the air sacs of dead and euthanized birds and cultured on 5% Columbia sheep blood agar according to the quadrant streaking technique. A semi quantitative estimate of *E. coli* isolation was conducted according to the growth on blood agar. Growth on these plates was recorded on a scale from 0 to 4+, where 0 = no growth; 1+ = growth of bacteria on area 1; 2+ = growth of the bacteria on areas 1 and 2; 3+ = growth of bacteria on areas 1, 2, and 3; and 4+ = growth of bacteria on areas 1, 2, 3, and 4(10).

Statistical analysis. Clinical scores of each bird for the 10 d period were summed to generate a cumulative clinical score (CCS) and the significance of differences among groups was ***tested*** using Kruskal Wallis nonparametric analysis of variance. The significance of difference in Survival analysis, bacteriological scoring and CCS were analyzed using Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA). The significance of differences among groups in survival patterns and median survival times were analyzed using the log-rank test and chi-square statistics.

RESULTS AND DISCUSSION

A significantly higher protection of neonatal broiler chickens against a lethal challenge of *E. coli* was noted by the IPL delivery of CpG-ODN as micro-droplets ($P<0.005$). The groups that received IPL CpG-ODN as micro-droplets had significantly lower CCS ($P<0.05$) compared to the group that received IPL saline as micro-droplets. Low counts of bacteria were isolated from the groups that received IPL CpG-ODN as micro droplets compared to IPL saline.

Disease prevention in terms of immune stimulation is more preferable than treatment in poultry industry due to a number of food safety and human health concerns. The use of therapeutic agents, particularly antibiotics by the animal industry has been linked to the emergence of antibiotic resistant bacteria as well as antibiotic residues in animal products (1). As a result, the animal production industry is seeking to reduce the use of therapeutic agents by developing effective immunotherapeutic molecules (5). The results of this experiment agreed with the previous discoveries of immunoprotective ability of CPG-ODNs administered in parenteral routes (6, 7), proving that IPL delivery could be an effective method of delivery of CpG-ODN in neonatal broiler chickens. Mortality due to bacterial infections of neonatal birds influences the remaining production cycle and growth of broiler chickens(13). It has been documented that mortality over 2% at the end of the first seven days of age is associated with poor growth, loss of uniformity and chronic infections within the flock and higher condemnations at processing (2). Because of these reasons, delivery of CpG-ODN by the IPL as micro-droplets at hatch is a valuable tool for disease prevention by immune stimulation. Since vaccine administration at the time of hatch is a common practice in the poultry industry, IPL delivery of CpG-ODN is an industry feasible method in the poultry industry. In conclusion, IPL micro-droplet delivery of CpG-ODN can protect neonatal broiler chickens against lethal bacterial infections at a significant level, ensuring welfare to the birds and food safety issues of consumers.

(The full-length article will be submitted to *Avian Diseases* shortly.)

REFERENCES

1. Agunos, A., D. Leger, and C. Carson Review of antimicrobial therapy of selected bacterial diseases in broiler chickens in Canada. The Canadian veterinary journal. La revue veterinaire canadienne 53:1289-1300. 2012.

2. Barnes, H., J. Vaillancourt, and W. Gross Colibacillosis. Diseases of poultry 12:691-732. 2003.

3. Brigden, J.L., and C. Riddell A survey of mortality in four broiler flocks in western Canada. The Canadian veterinary journal. La revue veterinaire canadienne 16:194-200. 1975.

4. Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J Antimicrob Chemoth 52:159-161. 2003.

5. Diarra, M.S., and F. Malouin Antibiotics in Canadian poultry productions and anticipated alternatives. Front Microbiol 5. 2014.

6. Gomis, S., L. Babiuk, B. Allan, P. Willson, E. Waters, N. Ambrose, R. Hecker, and A. Potter Protection of neonatal chicks against a lethal challenge of Escherichia coli using DNA containing cytosine-phosphodiester-guanine motifs. Avian diseases 48:813-822. 2004.

7. Gomis, S., L. Babiuk, D.L. Godson, B. Allan, T. Thrush, H. Townsend, P. Willson, E. Waters, R. Hecker, and A. Potter Protection of chickens against Escherichia coli infections by DNA containing CpG motifs. Infection and immunity 71:857-863. 2003.

8. Gyles, C.L. Antimicrobial resistance in selected bacteria from poultry. Animal Health Research Reviews 9:149-158. 2008.

9. H. John Barnes, L.K.N., Jean-Pierre Vaillancourt Colibacillosis. In: Diseases of Poultry, 12th edition ed. A.M.F. Y.M.Saif, J.R.Glisson, L.R.McDougald, L.K.Nolan, D.E.Swayne, ed. Blacwell Publishing Professional, Iowa. p 691. 2008.

10. Hoeprich, P.D. Infectious diseases; a guide to the understanding and management of infectious processes, 1st ed. Medical Dept., Hagerstown, Md.,. 1972.

11. Patel, B.A., S. Gomis, A. Dar, P.J. Willson, L.A. Babiuk, A. Potter, G. Mutwiri, and S.K. Tikoo Oligodeoxynucleotides containing CpG motifs (CpG-ODN) predominantly induce Th1-type immune response in neonatal chicks. Dev Comp Immunol 32:1041-1049. 2008.

12. Taghavi, A., B. Allan, G. Mutwiri, A. Van Kessel, P. Willson, L. Babiuk, A. Potter, and S. Gomis Protection of neonatal broiler chicks against Salmonella Typhimurium septicemia by DNA containing CpG motifs. Avian diseases 52:398-406. 2008.

13. Thushari Gunawardana, M.F., Tara Zachar, Shelly Popowich, Betty Chow-Lockerbie, Marina Vaneva Ivanova, Suresh Tikoo, Shanika Kurukulasuriya, Philip Willson, and Susantha Gomis Protection of neonatal broiler chickens following in ovo delivery of oligodeoxynucleotides containing CpG motifs (CpG-ODN) formulated with carbon nanotubes or liposomes. Avian diseases In press. 2014.

14. Yassin, H., A.G.J. Velthuis, M. Boerjan, and J. van Riel Field study on broilers' first-week mortality. Poultry Sci 88:798-804. 2009.

BROILER BREEDER VACCINATION WITH COMBINATION OF FOWL ADENOVIRUS (FADV)-8B AND FADV-11 INDUCES BROAD-SPECTRUM PROTECTION AGAINST IBH

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ABSTRACT

Inclusion body hepatitis (IBH), which is caused by several serotypes of fowl adenovirus (FAdV), is one of the most economically important diseases in broiler chicken industry. Currently used autogenous inactivated vaccines are associated with the unpredictable efficacy, and sometimes fail to prevent IBH in broiler chickens. The objectives of the present study were to evaluate the combination of FAdV8b and FAdV11 as live or inactivated broad-spectrum vaccines in broiler breeders. The immunogenicity of these vaccines was tested in broiler-breeders and protective efficacy was examined by broiler progeny challenge. Thirteen-week-old broiler breeders (n=300/group) were vaccinated either with a combination of live (FAdV)-8b and -11 vaccine via the oral route or by a combination of inactivated (FAdV)-8b and -11 vaccine via the intramuscular route. Another group, of age-matched breeders (n=150), was kept as unvaccinated control. The inactivated group was boosted three weeks later with respective vaccine. Sera samples were collected at 19, 22, 30 and 48 weeks of age and eggs were collected for progeny challenge. Neutralizing antibodies (NAbs) were detected at three weeks post-vaccination in both the groups and their level became significantly ($p<0.05$) higher for FAdV8b than FAdV11 at 22, 30 or 48 weeks of age in breeders vaccinated with live vaccine and at 20 and 48 weeks of age upon breeder vaccination with inactivated vaccine. NAbs response in breeders to either live or inactivated antigens of each virus were not significantly different ($p>0.05$). Irrespective of the type of vaccine, 98-100% protection ($p>0.05$) was observed in broiler progenies upon homologous virus challenge. FAdV8b and -11 induced cross-neutralizing antibodies to other serotypes of same species, thus this combination has potential to provide broad-spectrum protection against IBH in broilers.

INTRODUCTION

FAdVs cause a variety of infections in birds, but the most significant one occurs in broilers. These includes IBH, hepatitis-hydropericardium syndrome (HHS) and gizzard erosion and ulcers (GEU) (1). IBH is one of the major FAdV disease observed in Canadian chicken barns (5, 6). Numerous isolates have been identified that have close nucleotide identity to serotypes 2, 7, 8a, 8b and 11 of species E and D of FAdVs (10). The disease cause significant mortality among broilers that varies between 0.1 % to as high as 30 % in cases of IBH (11, 15) thus is lingering threat to broiler industry in the countries of their prevalence.

The disease is controlled by the use of autogenous inactivated vaccine [or live virus vaccine, only in Australia (7, 13)] in broiler breeders to ensure high maternal antibody transfer to progenies (2). Despite use of autogenous inactivated vaccines, sporadic outbreaks of diseases are reported time to time. Exposure to heterologous virus breaks the vaccinal immunity and contributes to the occurrence of IBH (13). Various researchers have reported lack of cross serum neutralization among FAdV species (8, 9) that likely explains the escape of heterologous viruses from vaccinal immunity. There is a need to evaluate a combination of heterologous viruses in a vaccine. Therefore, the goal of this study was to evaluate the combination FAdV8b and FAdV11 as live or inactivated vaccines in broiler breeders for their immunogenicity and protective efficacy against IBH in broilers. Live virus vaccines are economic and easy to use compared to inactivated vaccines, hence, a live

virus combination is included in the vaccine trial for comparative study.

MATERIALS AND METHODS

Viruses and cell line. FAdV-8b and FAdV11 were used in the study. Male white leghorn hepatoma (LMH) cell line was used to propagate and titer the virus.

Vaccine preparation. Live FAdV (FAdV8b/11) vaccine was prepared in sterile saline to make a dose of $1x10^4$ $TCID_{50}$ of each virus per bird. Similarly, heat inactivated viruses of known titer were mixed with Emulsigen-D and sterile saline to make a final dose of $1 x10^6$ $TCID_{50}$ of each virus in 20 % of Emusigen-D per bird.

Animal experiments. Day old (Ross) broiler breeders were purchased from Aviagen and were reared in animal care facility of Western College of Veterinary Medicine at University of Saskatchewan. Seven hundred and fifty broiler breeders were randomly divided into three groups (Group-1 and 2 = 300 birds, Group-3 = 150 birds), in the beginning of the experiment. Birds were bled at 15 weeks of age to test for anti-FAdV status. Following confirmation of FAdV-seronegative status, birds in the Group 1 were vaccinated with inactivated fowl adenovirus vaccine at 16 weeks and boosted three weeks later at 19 weeks of age. Birds in the Group 2 were vaccinated once with live vaccine at 16 weeks of age. Birds of the Group 3 were kept as unvaccinated control. Following vaccination, sera samples (n=10) were collected at 19, 22, 30, and 48 weeks of age and analyzed for neutralizing antibodies (Nabs) by micro neutralization test with some modification to the previous protocol (12). The eggs were collected from the breeders once they had sufficient antibody titers. The eggs were hatched and the chicks were challenged with FAdV8b and FAdV11 separately at 14 days post hatch.

Statistical analysis. Statistical analysis was performed in PRISM (GraphPad, Inc, USA). Differences in mean serological titers of breeders were tested using Student T –test with Welch's correction for unequal variance. Survival among progeny groups was analyzed by mantel cox (Log-rank) test. The tests were performed at a 95% level of significance (α= 0.05) and the results were considered significant at a p value < 0.05.

RESULTS

No antibody responses were seen before vaccination in any of the groups and in the control group throughout the experiment. The antibody production was evident three weeks post-vaccination in groups vaccinated with either live of inactivated combination vaccines. No significant differences were observed in the mean Nab levels of breeders to FAdV8b and FAdV11 at 19 weeks of age ($p>0.05$) following live vaccine. Thereafter, Nab levels steadily increased for FAdV8b at 22, 30 and 48 weeks of age. The NAb against FAdV8b were significantly higher than FAdV11 at 22, 30 and 48 weeks of age ($p<0.05$). Likewise, except at 19 weeks of age, mean Nab titers for FAdV8b were higher than FAdV11 following inactivated vaccine, and the differences were statistically significant at 30 and 48 weeks of age ($p<0.05$). Protective efficacy of broiler breeder vaccination in preventing IBH in their broiler progenies was tested at 14 days old broilers by lethal challenge with FAdV8b or FAdV11 by. Following challenge with FAdV8b, statistically significant survival ($p<0.05$) was observed in broilers hatched from the live (100% survival) or inactivated (98%) vaccinated breeders compared to control breeders. Similar to FAdV8b challenge, 98.8 % broilers survived lethal challenge with FAdV11 ($p<0.05$) from either of the vaccinated group.

DISCUSSION

Circulation of multiple FAdV serotypes that cause inclusion body hepatitis (3, 10) in Canadian chicken barns and unpredictable shift in their prevalence makes disease control difficult (14). To revise existing autogenous vaccines with a better one, a combination of cell culture propagated FAdVs (FAdV8b and FAdV11) were evaluated for immunogenicity in broiler breeders in live and inactivated format. Based on our findings, one prime with live FAdV (FAdV8/11) was sufficient to induce NAbs responses as early as three weeks that had amplified with age and remained consistent till the end of experiment. The levels of antibody responses were comparatively higher at all the time points against FAdV8b than FAdV11. Similar trend was observed in the kinetics of antibodies against the inactivated vaccine combination. This discrepancy in the level of antibody responses to each virus can be explained by the difference in the immunogenicity of the virus (4). Surprisingly, upon intergroup comparison of live and inactivated vaccine of same virus, no differences were observed for any of the virus. In progeny challenge studies, 98.8 to 100% protection was observed against FAdV8b or FAdV11, which highlights the essence of maternal antibodies against infection. In conclusion, broad-spectrum protection can be achieved by using combination of heterologous serotypes in live or inactivated format as they perform to the same level.

REFERENCES

1. Adair, B. M., and S. D. Fitzgerald. Group I Adewnovirus Infections. 12 ed. A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan and D. E. Swayne, eds. Blackwell Publishing. pp 252-256. 2008.

2. Alvarado, I. R., P. Villegas, J. El-Attrache, E. Jensen, G. Rosales, F. Perozo, and L. B. Purvis. Genetic characterization, pathogenicity, and protection studies with an avian adenovirus isolate associated with inclusion body hepatitis. Avian Diseases 51:27-32. 2007.

3. Choi, K. S., S. J. Kye, J. Y. Kim, W. J. Jeon, E. K. Lee, K. Y. Park, and H. W. Sung. Epidemiological investigation of outbreaks of fowl adenovirus infection in commercial chickens in Korea. Poultry Science 91:2502-2506. 2012.

4. Cottingham, M. G., A. van Maurik, M. Zago, A. T. Newton, R. J. Anderson, M. K. Howard, J. Schneider, and M. A. Skinner. Different levels of immunogenicity of two strains of Fowlpox virus as recombinant vaccine vectors eliciting T-cell responses in heterologous prime-boost vaccination strategies. Clin Vaccine Immunol 13:747-757. 2006.

5. Dar, A., S. Gomis, I. Shirley, G. Mutwiri, R. Brownlie, A. Potter, V. Gerdts, S. K. Tikoo, A. C. S. Gomis, B. I. Shirley, B. G. Mutwiri, A. R. Brownlie, A. A. Potter, A. V. Gerdts, and S. K. T. A. Pathotypic and Molecular Characterization of a Fowl Adenovirus Associated with Inclusion Body Hepatitis in Saskatchewan Chickens Pathotypic and Molecular Characterization of a Fowl Adenovirus Associated with Inclusion Body Hepatitis in Saskatchewan Chicke. 56:73-81. 2012.

6. Gomis, S., R. Goodhope, D. Ojkic, P. Willson, A. R. Goodhope, A. D. Ojkic, and P. W. C. Inclusion Body Hepatitis as a Primary Disease in Broilers in Saskatchewan , Canada Inclusion Body Hepatitis as a Primary Disease in Broilers in Saskatchewan , Canada. Avian Diseases 50:550-555. 2006.

7. Grimes, T. Dander. In. pp 1-14. 2007.

8. Kefford, B., R. Borland, J. F. Slattery, and D. C. Grix. Serological identification of avian adenoviruses isolated from cases of inclusion body hepatitis in Victoria, Australia. Avian Dis 24:998-1006. 1980.

9. McFerran, J. B., J. K. Clarke, and T. J. Connor. Serological classification of avian adenoviruses. Archiv fur die gesamte Virusforschungr 39:132-139. 1972.

10. Ojkic, D., E. Martin, J. Swinton, J.-P. Vaillancourt, M. Boulianne, and S. Gomis. Genotyping of Canadian isolates of fowl adenoviruses. Avian pathology : journal of the W.V.P.A 37:95-100. 2008.

11. Reece, R. L., D. a. Barr, D. C. Grix, W. M. Forsyth, R. J. Condron, and M. Hindmarsh. Observations on naturally occurring inclusion body hepatitis in Victorian chickens. Australian veterinary journal 63:201-202. 1986.

12. Schachner, A., A. Marek, B. Jaskulska, I. Bilic, and M. Hess. Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis-hydropericardium syndrome (HHS). Vaccine 32:1086-1092. 2014.

13. Steer, P. A., D. O'Rourke, S. A. Ghorashi, and A. H. Noormohammadi. Application of high-resolution melting curve analysis for typing of fowl adenoviruses in field cases of inclusion body hepatitis. Australian Veterinary Journal 89:184-192. 2011.

14. Venne, D. Field data of the changing clinical picture over time of IBH in Canada with an emphasis on diagnosis, prevention and trials on supportive treatments. In. pp 1-8. 2012.

15. Wells, R. J., H. A. Westbury, K. E. Harrigan, G. D. Coleman, and R. G. Beilharz. Epidemic adenovirus inclusions body hepatitis of the chicken in Australia. Aust Vet J 53:586-590. 1977.

THE EFFECT OF AN OREGANO-BASED FEED ADDITIVE (OREGO-STIM) ON THE DEVELOPMENT OF COCCIDIAL IMMUNITY AND BIRD PERFORMANCE DURING *EIMERIA* CHALLENGE

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SUMMARY

Orego-Stim (OS) has been shown to have positive effects on gut health. The interaction of OS in-feed and coccidial immunity was studied. A total of 160 Cobb 500 birds were spray-vaccinated at day-old with an anticoccidial vaccine and allocated to four treatments (40 birds/treatment; eight replicates/treatment): R) Robenidine 33g/T; O1) OS 150g/T; O2) OS 300 g/T; O3) OS 450g/T. Birds were floor reared on reused litter until day 21, moved to cages and challenged orally with a mixed *Eimeria* infection. Coccidia-free birds were used as Positive Control (PC) (five birds/cage, eight replicates). On day 27 birds were euthanized and coccidial lesion scored. Lesion scores were lower with OS1, OS2, OS3 and R versus PC: PC: 2.8, R: 1.5, O1: 1.2, O2: 1.0, O3: 1.1 ($p<0.001$). Bodyweight gains from days 21-27 were higher with OS treatments: PC: 0.082, R: 0.157, O1: 0.227, O2: 0.215, O3: 0.222 kg/bird ($p<0.001$). This study demonstrated that Orego-Stim improved bird performance during coccidial challenge and did not disrupt the development of coccidial immunity.

INTRODUCTION

Eimeria is the main protozoal disease affecting poultry globally. Coccidiosis is estimated to cost the US poultry industry $127 million annually (1). Antiprotozoal drugs and vaccines have long been used in commercial poultry production but there are no new compounds coming to market. With the withdrawal of some existing products as well, the tools to help manage protozoal poultry infections are becoming limited. Feed additives, such as those based on essential oils are increasingly used in poultry production to support both antibiotic free and conventional production. However, reports on efficacy of essential products against coccidia under severe field challenge are limited and questions remain whether such products interfere with the development of coccidial immunity, for example following coccidiosis vaccination. The current study was intended to determine if this commercial oregano-based product (Orego-Stim) would interfere with the development of immunity to *Eimeria*.

METHOD

The study was conducted in the facilities of Southern Poultry Research, Inc, Athens, GA, USA. A total of 160 Cobb 500 male birds were used in this challenge phase of the study. Birds were vaccinated at day-old with Coccivac B-52 (Merck, USA) via hatchery spray and allocated to four treatment groups (40 birds/treatment, eight replicates/treatment). R) Basal diet incorporating Robenidine (Robenz, Zoetis, USA), 33 g/T; O1) Basal diet containing Orego-Stim (Anpario Plc, UK), 150g/T; OS2) Basal diet containing Orego-Stim 300g/T; OS3) Basal diet containing Orego-Stim 450g/T. All basal diets were free of anticoccidial additives other than those described for the treatment allocation. Birds were housed on reused litter and provided feed and water *ad libitum*.

Birds were floor reared on reused litter until day 21, moved to cages and challenged orally with a mixed *Eimeria* infection. Coccidia-free birds were used as Positive Control (PC) (five birds/cage, eight replicates). Birds were fed basal diets free of all additives. All birds were challenged orally with a 1 mL dose containing 100,000 oocysts of *Eimeria acervulina*, 40,000 oocysts of *E. maxima* and 75,000 oocysts of *E. tenella*. On day 27 birds were euthanized and intestinal tracts examined and scored for coccidial lesions (2).

Bodyweight was recorded on day 21 and day 27. Data were analysed using ANOVA using Minitab v17 (Minitab Inc, USA).

RESULTS

There was a significant effect of treatment upon bodyweight gain (BWG) (Table 1). PC birds had significantly lower BWG compared to the other treatment groups. PC had a minimum 0.075 kg lower

BWG than the nearest treatment, group R. OS1 and OS3 had significantly higher BWG than PC and R. BWG was highest in OS1. Mean BWG in all OS groups was 0.064 kg greater than R and 0.139 kg greater than PC

Lesions scores were significantly affected by treatment (Table 1). Compared to PC, all other treatments had significantly lower mean lesions score for *E. acervulina*, *E. maxima* and *E. tenella*. Consequently mean lesion score was significantly higher in PC compared to R and OS treatments. Lesion score did not differ significantly between R, OS1, OS2 and OS3. OS2 had the lowest mean lesion score (1.03). The highest average reduction in lesion score was observed with the *E. maxima* challenge, where the average reduction in lesion score of treatments versus PC was 68%.

DISCUSSION

The study was designed to evaluate the effect of Robenidine (Robenz) and Orego-Stim on the development of immunity to *Eimeria*. The hypothesis was that Robenz, a highly effective anticoccidial chemical, would prevent oocyst cycling to a greater extent than Orego-Stim, resulting in higher lesion scores and lower bodyweight gain compared to Orego-Stim.

The high lesion scores and low bodyweight gain observed in the Positive Control group confirmed that the *Eimeria* challenge offered considerable intestinal challenge for the birds. Robenz was highly effective at mitigating the impact of *Eimeria* damaging the intestinal lining and consequently an increase in bodyweight gain compared to the positive control. However, bodyweight gains and lesion scores were improved further in all the Orego-Stim treatments compared to Robenz, confirming the hypothesis that an oregano-based product does not interfere with the development of coccidial immunity, for example following coccidiosis vaccination at day-old. Previous work using a similar challenge model to that used in the current study demonstrated improved performance using Orego-Stim (450 g/T) compared to an anticoccidial vaccine over a 42 day period and comparable coccidial control to both vaccine and salinomycin following *Eimeria* challenge at 21 days (3). Furthermore, the study demonstrated a synergistic effect of combining the oregano product with the anticoccidial vaccine for both improved bird performance and coccidial lesion score reduction. An oregano-based product has also been demonstrated to confer a reduction in lesion score similar to Diclazuril (4). However, in that instance, the original challenge dose resulted in lesion scores of approximately one, substantially lower than the challenge model used in the current study. The challenge dose selected in the current study was designed to maximise the adverse effect on performance and intestinal health to mimic a field challenge.

There was no apparent linear relationship between Orego-Stim inclusion level and either lesion score reduction or bodyweight gain. While thymol and carvacrol have been shown to directly damage the oocyst cell wall *in vitro* (5), this is unlikely to be the primary mode of action in the animal due to low oil concentrations in the feed. Most likely, the apparent anticoccidial activity of oregano is based up on a number of factors including, immunomodulation (6), gut microbiota modulation (7) and stimulation of enterocyte proliferation (8). Robenidine, however, is known to act directly on the *Eimieria* life stages, for example first and second stage schizonts (9).

CONCLUSION

The present study clearly demonstrated that the inclusion of Orego-Stim in-feed did not interfere with the development of immunity to *Eimeria*. In light of the development of resistance in *Eimeria* populations to anticoccidial drugs and a move to towards the reduction of such compounds in some areas of poultry production, natural feed additives such as Orego-Stim have a significant role to play in coccidiosis management programmes. Furthermore, such oregano-based products could also be used to support anticoccidial vaccination programmes.

REFERENCES

1. Chapman, H. D. A landmark contribution to poultry science – prophylactic control of coccidiosis in poultry. Poult. Sci. 88:813–815. 2009.

2. Johnson, J. and W. M. Reid. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Exp. Parasitol. 28:30-36. 1970.

3. Harrington, D., K. Konstanti, G. F. Mathis and L. Broom. Oregano essential oil improves performance in coccidia vaccinated broilers. Abstract. Proc. Poult. Sci. 95(E-Suppl. 1): pp. 82. 2016.

4. Mohiti-Asli, M. and M. Ghanaatparast-Rashti. Dietary oregano essential oil alleviates experimentally induced coccidiosis in broilers. Prev. Vet. Med. 120:195–202. 2015.

5. Remmal, A., S. Achahbar, L. Bouddine, F. Chami and N. Chami. Oocysticidal effect of essential oil components against *Eimeria* oocysts. Int. J. Vet. Med. Res. Rep: 2013:pp. 8. 2013.

6. Mohiti-Asli, M. and M. Ghanaatparast-Rashti. Comparison of the effect of two phytogenic compounds on growth performance and immune

response of broilers. J. Appl. Anim. Res. 45:603–608. 2016.

7. Roofchaee, A., M. Irani, M. A. Ebrahimzadeh and M. R. Akbari. Effect of dietary oregano (*Origanum vulgare* L.) essential oil on growth performance, cecal microflora and serum antioxidant activity of broiler chickens. Afr. J. Biotechnol. 10:6177–6183. 2011.

8. Levkut, M., A. Marcin, V. Revajová, L. Lenhardt, I. Danielovič, J. Hecl, J. Blanár, M. Levkutová and J. Pistl. Influence of oregano extract on the intestine, some plasma parameters and growth performance in chickens. Acta. Vet. Beogard. 61:215–225. 2011.

9. Kant, V., P. Singh, P. K. Verma, I. Bais, M.S. Parmar, A. Gopal and V. Gupta. Anticoccidial drugs used in poultry: An overview. Sci. Int. 1:261–265. 2013.

Table 1. Mean bodyweight gain (BWG) from 21-27 days and mean lesion score according to *Eimeria* species and overall lesion score on day 27.

Treatment	BWG (kg)	Day 27 Lesion score			
		E. acervulina	E. maxima	E. tenella	Mean
PC	0.082[c]	3.03[a]	2.39[a]	2.91[a]	2.78[a]
R	0.157[b]	1.63[b]	1.20[b]	1.70[b]	1.51[b]
OS1	0.227[a]	1.33[b]	1.03[b]	1.20[b]	1.18[b]
OS2	0.215[ab]	1.25[b]	0.88[b]	0.98[b]	1.03[b]
OS3	0.222[a]	1.23 [b]	0.80[b]	1.28[b]	1.10[b]
SEM	0.0181	0.119	0.113	0.142	0.119
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

Values within a column not sharing a common superscript differ ($P < 0.05$).

OMEGA-3S REDUCE CAMPYLOBACTER IN BROILER CHICKENS

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SUMMARY

Campylobacter causes thousands of human intestinal infections annually. It is estimated that 50-80% of these cases are attributed to chicken consumption. Omega-3 fatty acids are recognised as inflammatory mediators and inflammation is considered to be a factor in intestinal *Campylobacter* colonization. Therefore, increasing the omega-3 content of broiler chicken diets may reduce *Campylobacter* colonization. A series of trials were conducted to investigate the role of omega-3s (Optomega 50) in controlling broiler *Campylobacter* levels. Optomega 50 reduced cecal *Campylobacter* numbers ($P<0.001$) by up to 99%. Optomega 50 was most effective if supplemented from hatch, with an optimum dietary inclusion found to be 1.0%. These studies show that Optomega 50 can be used effectively to reduce *Campylobacter* carriage in poultry.

INTRODUCTION

Poultry is a major reservoir and source of transmission for *Campylobacter* in humans, most notably attributed to *Campylobacter jejuni*. *Campylobacter* is a major cause of enteritis globally (1) although the estimated rate of campylobacteriosis differs strongly with by country and region. A number of interventions have been considered for poultry production pre- and post-harvest to lower *Campylobacter* carriage including thinning poultry flocks mid production and surface chilling carcasses. High levels of *Campylobacter* in the avian intestine are a source of environmental contamination on the farm. Reducing bacterial numbers in the bird would provide a complementary approach to existing intervention practices.

Campylobacter uses host inflammatory responses to colonize the intestine (2). Reducing intestinal inflammation may therefore lower *Campylobacter* colonization. Polyunsaturated fatty acids (PUFAs) such as omega-3 have been shown to have anti-inflammatory effects. Optomega 50 (Anpario, UK) comprises salmon oil rich in omega-3s, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Two studies were undertaken to determine if the addition of Optomega 50 to broiler diets could lower the incidence numbers of *Campylobacter* in birds inoculated with the organism.

METHOD

Ross 308 day-old broiler chicks, obtained from a local commercial hatchery, were used in two experiments. In experiment one, birds were fed standard commercial diets containing 0, 0.25, 0.35, 0.45, 0.55 and 0.75% salmon oil (Optomega 50, Anpario, UK), with salmon oil levels halving from day 25 to slaughter. Optomega 50 is 50% salmon oil and 50% carrier; hence Optomega 50 inclusions are twice the stated salmon oil inclusions. Birds were orally inoculated with a 10^5 dose of *Campylobacter jejuni* M1 suspended in Mueller Hinton Broth at 14 days of age. There was also an uninfected, non-supplemented control. Fifteen birds per group were humanely euthanized at 35 days of age and the ceca obtained for *Campylobacter* analysis. In experiment two, birds were fed a standard, non-supplemented control diet from hatch and infected with a 10^5 dose of *C. jejuni* M1 at 21 days of age. At 28 days of age (seven d.p.i.), half of the birds were transferred to a 0.5% salmon oil diet (Optomega 50, Anpario, UK), while the remainder stayed on a non-supplemented control diet. At 35 days of age, fifteen birds per group were humanely euthanized to obtain cecal samples for *Campylobacter* analysis.

Birds were stocked at commercial density and had constant access to food and water. Cecal samples were serially diluted and spread plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) and these plates were incubated at 37 °C for 48 hours under microaerobic conditions consisting of 5% O_2, 10% CO_2, 5% H_2 and 80% N_2.

Differences in bacterial colonization were analysed using a one way ANOVA with a Tukey's post test (GraphPad Prism 5 software). Significance was declared at $P<0.05$.

RESULTS

Experiment 1. At 21 d.p.i, a reduction in *Campylobacter* levels was seen in all diets containing

0.35% salmon oil (0.7 % Optomega 50) and greater (Figure 1).

Experiment 2. Birds infected at 21 days and fed 0% Optomega 50 until 28 days followed by 0.5% salmon oil/1% Optomega thereafter had similar numbers of *Campylobacter* in their caecum compared to control birds fed 0% Optomega 50 throughout.

DISCUSSION

Poultry is generally colonized by *C. jejuni* at approximately two weeks of age (3). Despite extensive intestinal colonization, chickens usually do not show overt signs of illness, although weight loss, diarrhea and mortality have been recorded in infected poultry. Poultry is considered the main source of human campylobacteriosis worldwide (1). *Campylobacter* control has focused on both pre and post-slaughter approaches. On-farm control generally falls into three general strategies: biosecurity, use of antimicrobial alternatives e.g. bacteriocins or bacteriophages, and increase host resistance e.g. competitive exclusion and vaccination (4). These strategies have had variable effectiveness. Given the avian intestine is the main reservoir and point of amplification of *Campylobacter* in the food chain, on-farm control, including in the bird itself, would have the greatest impact (4)

Campylobacter acts synergistically with the proinflammatory cytokine IFN-γ to break down tight junctions between cells (2) and thereby circumvent the intestinal epithelial barrier. This behaviour is thought to set up a feedback loop of inflammation, allowing high numbers of *Campylobacter* across the epithelium (5). This allows the colonization of internal organs such as the liver, and is also a source of re-infection of the intestine from a protected replication niche within the tissue. PUFAs, particularly omega-3s have been shown to increase and enhance the intestinal barrier and have anti-flammatory effects (6, 7). Birds are poor converters of dietary omega-3s into EPA and DHA, estimated at 5%, hence feeding a source high in EPA and DHA such as Optomega 50 should increase EPA and DHA bioavailability in the bird.

In the current study, infecting birds fed with Optomega 50 from day-old with *Campylobacter* at 14 days lowered *Campylobacter* numbers in the caecum. However, if birds were infected prior to the inclusion of Optomega 50 in the feed, the level of *Campylobacter* infection was not lowered. These data would suggest that feeding a source rich in EHA and DPA is able to lower the level of cecal colonization by *Campylobacter* but is less effective once the infection has established. Since *Campylobacter* utilises the host inflammatory response for colonization, it follows that modulation of the pro-inflammatory response post colonization would be less effective to eliminate an established bacterial infection.

CONCLUSION

Optomega 50 fed to birds at 0.35% or greater from day-old lowered cecal Campylobacter levels in infected poultry, although it was necessary for Optomega 50 to be included in feed prior to the establishment of a *Campylobacter* infection. Feeding omega-3 PUFAs could have potential as part of an on-farm strategy for *Campylobacter* control.

REFERENCES

1. Kaakoush, N. O., N. Castaño-Rodriguez, H. M. Mitchell and S. M. Man. Global epidemiology of *Campylobacter* infection. Clin. Microbial. Rev. 28:687–720. 2015.

2. Rees, L. E., T. A. Cogan, A. L. Dodson, M. A. Birchall, M. Bailey and T. J. Humphrey. *Campylobacter* and IFNgamma interact to cause a rapid loss of epithelial barrier integrity. Inflamm. Bowel. Dis. 14:303–309. 2008.

3. Evans, S. J. and A. R. Sayers. A longitudinal study of *Campylobacter* infection of broilers in Great Britain. Prev. Vet. Med. 10:209–223. 2000.

4. Jun, L. Novel approaches for *Campylobacter* control in poultry. Foodborne Pathog. Dis. 6:755–765. 2009.

5. Chen, M. L., Z. Ge, J. G. Fox and D. B. Schauer. Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*. Infect. Immun. 74:6581–6589.

6. Donnet-Hughes A., E. J. Schiffrin and M. E. Turini. The intestinal mucosa as a target for dietary polyunsaturated fatty acids. Lipids 36:1043–1052. 2001.

7. Whiting, C. V., P. W. Bland and J. F. Tarlton. Dietary n-3 polyunsaturated fatty acids reduce disease and colonic proinflammatory cytokines in a mouse model of colitis. Inflamm. Bowel. Dis. 11:340–349. 2005.

Experiment 1. Effect of salmon oil inclusion level (%) on cecal *Campylobacter* levels.

INACTIVATION OF HIGH AND LOW PATHOGENIC AVIAN INFLUENZA VIRUSES IN FOOTBATHS AND THEIR PERSISTENCE IN POULTRY MANURE

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SUMMARY

A highly pathogenic avian influenza virus (HPAIV) H5N8 was introduced by migratory birds from Asia to North America during late 2014. In California, two outbreaks in commercial poultry were caused by this virus in January and February 2015 and a reassorted virus (HPAIV H5N2) spread throughout the Midwestern region via the Mississippi and central flyways, causing unprecedented losses mostly in the Midwestern region. It is very likely that the virus was introduced into new areas by wild birds, but secondary spread occurred along the industry contact networks.

Disinfectant footbaths are widely used as biosecurity measures against introduction of avian influenza and other viruses; however their use obviously was not effective in a number of cases during the HPAI outbreak. In the present study we collected information about footbath preparation and maintenance from poultry premises in California by an online survey. Ten individual responses representing small independent farms as well as large scale broiler, turkey and layer producers were obtained. Most commonly used disinfectants were quaternary ammonia, quaternary ammonia in combination with glutaraldehyde, phenol, iodine and bleach. Bleach was used either in liquid or powder form. Frequency of preparation, location of footbath as well as the requirement of scrubbing boots varied between the respondents.

The information was used to design an experiment to examine footbath disinfection effectiveness. Three footbaths based on quaternary ammonia, quaternary ammonia combined with glutaraldehide and bleach powder were prepared following manufacturer's specifications. A mix of layer feces, turkey and broiler bedding was spiked either with an HPAIV (H5N8) outbreak isolate or, for comparison, with a low pathogenic avian influenza virus (LPAIV H6N2) isolate. Rubber boot soles and especially their crevices were covered with the spiked mix and then dipped for three seconds into the footbaths directly after their preparation as well as 24, 48 and 72 hours after being prepared. A freshly prepared spiked boot was used per each footbath tested and every day a new batch of spiked material was prepared. After eliminating excess material from the sole surface, samples were collected from the boot crevices. Viral load was quantified by RT-qPCR and re-isolation of the viruses was attempted in embryonated specific pathogen free (SPF) eggs. Both viruses were detected by RT-qPCR in all samples taken from boots treated with quaternary ammonia only or quaternary ammonia in combination with glutaraldehide; there was no correlation between viral load and age of the footbath. The viruses were also re-isolated from all these samples. In contrast, the bleach powder footbath decreased the viral load below the detection limit of the RT-qPCR at all sampling times and re-isolation was not possible.

Even though it was shown in other investigations that quaternary ammonia and quaternary ammonia in combination with glutaraldehide are in principle capable of inactivating AIV, footbaths based on these disinfectants were not effective under the tested settings. Obviously the disinfectants did not penetrate the organic matter sticking to the boot in the crevices. Additionally, a relatively high titer used for spiking and a short immersion in the footbath without scrubbing the boots created a worst-case scenario. However, comparable titers have been detected in excretions of infected birds, and the majority of responders in the survey stated that they would not require scrubbing the boots when using the footbaths.

The apparent effectiveness of the dry bleach might be due to a carry-over of bleach granules into the media tube during sampling, when dissolving the bleach granules in the small volume of sampling media might have led to high bleach concentrations inactivating the virus after the actual sampling.

The age of the footbaths was not a factor, since even freshly prepared footbaths containing quaternary

ammonia or quaternary ammonia and glutaraldehide were ineffective while aged dry footbaths with bleach still were capable to inactive the viruses.

In another experiment, layer feces as well as broiler and turkey bedding material were spiked either with HPAIV or LPAIV. Every 12 hours, starting at zero and ending at 96 hours after spiking, samples were investigated by RT-qPCR and virus re-isolation was attempted. Throughout the experiment viral RNA was detected in all samples. For both viruses, broiler bedding material showed the highest viral load levels, followed by layer feces and turkey bedding material. HPAIV stayed infective for less than 60 hours in broiler and turkey bedding, while in layer manure HPAIV it persisted until the end of the experiment 96 hours post spiking. LPAIV was re-isolated from layer feces, broiler and turkey bedding material only up to twelve hours after spiking.

HPAIV could be re-isolated for more than four days from layer feces. This high tenacity in layer feces might have contributed to the rapid and wide spread in layer facilities in 2015. Several causes like the number of cycles the litter had been used or different litter treatment between cycles with amendments or acidifiers might have contributed to the pronounced differences between the different bedding materials and feces. More systematic research would be needed in order to determine the influence of the specific variables.

In our investigation HPAI stayed longer infective than LPAI. In contrast to that, a previous investigation showed that LPAI H5 and H7 viruses were more persistent in water than HPAI H5 and H7 viruses. The superficially conflicting results can be explained by the adaption of LPAI to circulation in water fowl, for which a prolonged infectivity in water is necessary.

In conclusion, the high tenacity of the HPAIV strain and the ineffectiveness of most of the tested footbaths under the tested conditions help to explain the extent of the 2015 HPAI outbreak.

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

CHARACTERISTICS OF ANTIMICROBIAL RESISTANCE IN *SALMONELLA* SEROTYPES FROM ENVIRONMENTAL SAMPLING OF BROILER FARMS

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ABSTRACT

Mississippi State University Poultry Research and Diagnostic Laboratory was approached to conduct a field study to determine an appropriate application of environmental sampling techniques using boot swabs verses organ (ceca and spleen) samples commonly collected to survey a flock for *Salmonella* prior to processing. Environmental samples were collected using sterile boot swabs. The collection of environmental samples from five broiler farms were cultured, isolated, serotyped and sensitivities were evaluated. This study focuses on the similarities and differences of antimicrobial resistance from 50 day old broiler flock environments at five separate farms within one broiler company. Samples were isolated from *Salmonealla* serotypes Braenderup, Kentucky, and Enteriditis in work performed by Pulido Landínez et al using ISR assigned serotyping extraction method. Culture and sensitivities were performed on the environmental samples. WHONET was utilized to enhance the antimicrobial resistance of the samples.

INTRODUCTION

With the demand on the poultry industry to produce a safe produce for consumers the details surrounding different *Salmonella* serotypes are commonly evaluated. The poultry industry needs to be resourceful when determining the potential of foodborne bacteria entering the processing plant and possibility the human food supply. Environmental sampling is used commonly in the layer industry to determine the presence of *Salmonella* but have been utilized less in the broiler industry. The use of boot swabs is a tool that can assist in the determination of the presence and strains of *Salmonella* in broiler houses before the flock goes to harvest. Once there is a determination of the *Salmonella* serotype an evaluation of microbial sensitivities can also provide useful data. Antimicrobial resistence continues to be a growing concern in both human and animal medicine. The use of information gathered from environmental samples in a poultry facility can be used to not only monitor but show similarities and differences of bacterial profiles within the same environment.

MATERIALS AND METHODS

Boot swabs from Qualicum Scientific Ltd/Solar Biologicals Inc. pre-moistened cotton-poly blend fabric sock style boot covers were used in 11 poultry broiler houses. The same collector walked from the center entrance to each end of poultry house. Both boot swabs were removed and place in a whirl bag with approximately 100 mL of Buffered Peptone Water and stored in a cooler with icepacks for transportation. The National Poultry Improvement Plan (NPIP) procedure for collection, isolation, and identification of *Salmonella* from house environmental samples, cloacal swabs, and hatchery samples, subpart B - isolation and identification of *Salmonella* guidelines were followed (1). Serotyping of the Intergenic Sequence Ribotyping (ISR) *Salmonella* strains were submitted to eurofins|Genomics in Louisville Kentucky.

TREK Diagnostic Systems Sensititre® AVIAN1F MIC plates were utilized to determine the sensitivities of the serotyped *Salmonella* serotypes. The AVIAN1F MIC plate includes the following antibiotics: enrofloxacin, gentamicin, ceftiofur, neomycin, erythromycin, oxytetracycline, tetracycline, amoxicillin, spectinomycin, sulphadimethoxine, trimethoprim/sulfamethoxazole, florfenicol, sulphathiazole, penicillin, streptomycin, novobiocin, tylosin tartrate and clindamycin. Collected data were tabulated and evaluated using WHONET.

RESULTS

Assessment of the data collected using WHONET will be further evaluated.

REFERENCES

1. United States Department of Agriculture, Animal and Plant Health Inspection Service,

Veterinary Services. National Poultry Improvement Plan Program Standards. Aug 2014.

THE CHALLENGES AND FUTURE OF DIAGNOSTICS IN POULTRY MEDICINE

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OVERVIEW

A review of the past helps to frame the future of poultry diagnostics**.** In the United States, the Land-Grant universities (Morrill Act, 1862) provided a fertile environment for faculty members in the early 20th century to apply the scientific method to poultry disease diagnosis (8,9). A natural affiliation and partnership of the USDA Bureau of Animal Industry and state departments of agriculture developed with these fledgling diagnostic programs. A mutual need existed for reagents and testing to support programs for tuberculosis, hog cholera, and brucellosis, among others. For poultry, this era marked the launch of the National Poultry Improvement Plan (NPIP) in the 1930s to control Pullorum disease (2).

The poultry diagnostic expertise in the Land-Grant universities lead to the characterization of important poultry diseases in the years leading up to World War II: Newcastle disease, infectious bronchitis, mycoplasmosis, coccidiosis and others. The first printing of Diseases of Poultry appeared in 1943. Parallel to these activities was the formation of the United States Animal Health Association in 1897, from which was formed the American Association of Veterinary Laboratory Diagnosticians (AAVLD) in 1957, coinciding with the founding of the American Association of Avian Pathologists (AAAP) that same year. Through the 1960s and 70s, state and federal diagnostics were substantially moved from research laboratories into dedicated facilities. Their core mission was disease regulation, but general diagnostic services were provided for animal industries, including poultry. Private sector laboratories also were in operation during this time, some as technical service extensions of pharmaceutical and biological companies serving the poultry sector.

The state and university diagnostic laboratories were also the first line of support for the emerging field of companion animal medicine. A natural tension often developed within this setting because of finite diagnostic resources need to cover regulatory programs, animal industries, and companion animals as well as public health, wildlife and various other added services and programs. This was accentuated by the common practice of offering free or reduced-cost diagnostic services for agriculture to increase passive surveillance for regulated diseases. In this competitive environment, the amount of resources dedicated to poultry, state-by-state, was often contingent upon poultry producers who could leverage the largesse of university administrators and state agricultural authorities. Animal disease emergencies (Pullorum disease, chronic respiratory disease, tuberculosis, Newcastle disease, influenza, chronic wasting disease, concerns of agroterrorism) often provided the stimuli for renewed investment in diagnostic services for the animal industries.

Poultry diagnostics today is spread across multiple organizational structures. Necropsy examination and much specimen collection occurs on the farm. The most comprehensive diagnostic capabilities reside in state and university-affiliated laboratories in various administrative configurations. These labs refer cases to National Veterinary Services Laboratory (NVSL) as needed, and serve as nodes on the National Animal Health Laboratory Network (NAHLN). Government and university research laboratories provide diagnostics that are more narrowly defined and reflect the lab mission or the interests of the investigator. Private sector poultry diagnostics variously provide microbiology, serology, pathology and toxicology services. Poultry production company laboratories provide quality assurance testing of incoming feed ingredients, NPIP testing, and support of the processing plant, among other services.

Advances in poultry diagnostics stem chiefly from academic and corporate research laboratories, and from diagnostic laboratories. Quality system implementation has permeated poultry diagnostics in recent decades through AAVLD laboratory accreditation, membership in the NAHLN, and corporate quality programs, all based on standards defined by the International Organization for Standardization (ISO). This trend is sure to continue because it promotes trust among diagnosticians, producers, customers, and regulators worldwide.

TRADITIONAL POULTRY DIAGNOSTICS

A traditional diagnostic investigation is a response to problem, be it mortality, impaired production parameters, or a spike in losses at processing. The occurrence of a single disease such as influenza or a novel viral neoplasia can be highly significant. More often, the problem is multifactorial.

A diagnosis of coccidiosis is useful, but for commercial poultry, there may be contributing diseases (enteritis, immunosuppression), or factors other than disease (litter mismanagement, drug resistance and coccidia vaccination errors), that serve to promote the coccidiosis problem. Thus, poultry diagnostics requires not just a knowledge of a disease process but an understanding of the poultry production system. Excluding regulatory tests that require a positive or negative result, poultry diagnostics requires identification of the presenting disease and the contributing diseases, and formulating a collective pathogenesis as the basis of control and prevention. It depends on the experience of the diagnostician to rank the duration and severity of each component.

A challenge in poultry diagnostics is first, for the right information to be generated and second, delivered to the right person. The weakness becomes evident when the diagnostician is unable to investigate deeply enough because of technical, economic, or intellectual restraints. Conversely, the recipient of diagnostic information may be unable to understand the interrelationships or to take corrective action. Traditional diagnostics can be repetitive, with many similar cases submitted and investigated, and resources utilized to arrive at the same endpoint. The lower the fee for service, the more likely this is to occur. The captured data however remains valuable if uniformly recorded and analyzed for epidemiological trends.

PRODUCTION-INTEGRATED DIAGNOSTICS

Another way to investigate disease interactions is to integrate diagnostics with production. This model is based on methods used in toxicologic pathology, which uses laboratory animals of identical genetics raised with uniform housing and nutrition, thus comprising a homogenous population that is sampled at defined ages to assess treatment effects. This model closely resembles integrated commercial poultry production. The poultry population (complex, farm, or house) can be sequentially sampled over time for lesion identification and quantitative (measurements, counts) and semiquantitative (lesion scores) characterization (Fig. 1). It identifies the age of onset for collection of samples for etiologic agent detection, and thereby guides mitigation. It opens the process to better understanding of subclinical factors contributing to poor uniformity, at-risk subpopulations, and erosion of genetic potential and welfare. The numerical data, properly analyzed, provides visual representation that invites input from the perspective of health, nutrition, and management. It lends to comparative analysis over time because it is subject to quality management procedures for assessment.

Integrated poultry diagnostics can begin with on-site data collection of necropsy findings, supported by analytical software (Elanco Health Tracking System™, Indianapolis, IN; VDP PathPro©, Fort Valley, VA). This can be supplemented with a wide range of analyses. Quantitative and semiquantitative histopathology provides understanding of lesion definition, development, and impact. Sample collection for serology and infectious or toxicological agent characterization yields data to correlate with lesion development, with defined age of onset and severity. Production-integrated diagnostics adds value to the decision-making process.

Production-integrated pathology can help define the relationship of the gut biome to anatomical development and pathological degradation of the gut mucosa. It can define the sequence of immunosuppressive diseases and their interactions through analysis of multiple systems at different ages. Integrated pathology can be applied to critical events in skeletal development, as well as provide correlates for wellness examinations involving footpad quality and gait assessment. In mature poultry, it has found application in mortality surveys and reproductive assessment at various ages.

DIAGNOSTICS IN THE POST-ANTIBIOTIC ERA

The ongoing reduction in antibiotic usage and the emergence of the nutraceuticals (direct fed microbials, pre- and postbiotics, fermentation products, botanical extracts) create a challenge for bacterial diagnostics. To a considerable extent, nutraceuticals are in development as substitutes to achieve the health benefits derived by antibiotics (6). Antibiotics have defined absorption, distribution, and excretion patterns and specific modes of action. Bacterial isolates are routinely tested for antibiotic sensitivity to guide judicious usage and successful therapy. From a regulatory perspective, nutraceuticals in general are on the FDA Generally Recognized as Safe (GRAS) list. Although some have data for *in vitro* activity against poultry pathogens, claims of treatment efficacy are purposely absent, as opposed to regulated antibiotics. Trial data replaces registered claims in demonstrating comparable effects to antibiotics or antibiotic-drug combinations. Compared to antibiotics, the mechanisms of action and interactions of the bioactive components, their distribution and spectrum of activity may have scant to extensive characterization.

The nutraceuticals are coming onto the poultry market at the same time as high-throughput

sequencing and metagenomic analysis of the gut microbiome are becoming mainstream in poultry research laboratories (4). An inherent issue with nutraceuticals is the consistency of beneficial results in the production environment. Diagnostic laboratories may have a role in refining the process. Suppression of *Clostridium perfringens, Salmonella,* and *Campylobacter* is a desired outcome, as well as counteracting dysbacteriosis through modulating and maintaining a healthy balance of gut bacterial for optimum production efficiency. The bacterial populations are assessed by metagenomic analysis rather than traditional culture methods. This will require an expansion of capability for traditional bacteriology laboratories. Many of the botanical extracts show inhibitory effects *in vitro*, but standardized testing as applied to antibiotic sensitivity is less understood. The digestive tract is the logical first application; however, substantial new information would be needed to assess therapeutic possibilities for septicemia and deep tissue infections.

METAGENOMIC ANALYSIS AND ORGAN-SPECIFIC MICROBIOTA

The gut microbiome is now generally recognized to include viruses, and high-throughput sequencing has shown that more are present than previously recognized (1). This confirms diagnostic detection of multiple viruses in cases of viral enteritis, including astrovirus, rotavirus, reovirus, parvovirus (5). Others viruses become apparent with metagenomic analysis, such as picornaviruses and bacteriophages. While the concept of one virus acting as a primary pathogen remains valid, the respective identities, tissue loads, and interactive virulence of these viruses as enteric pathogens will continue as an emerging story. Certain bacterial and viral taxa show the ability to shift together in dominance within the microbiota (1). Effacement of the gut mucosa caused by parasites and toxins further affect the balance of the microbiota, the manner of which is just now being revealed. The microbiomes of poultry skin (carcass rinse) (3), and respiratory (7)**,** urogenital, and skeletal systems invite research investigation and diagnostic applications, and can be anticipated to find a place in the poultry diagnostic lexicon.

DISCUSSION

In addition to traditional state and university-based diagnostic laboratories, there are opportunities to provide diagnostic services through an emerging private sector. While poultry consultants have long played a role in poultry health, privately-owned, independent diagnostic service providers create additional options for those needing services, as well as those seeking careers n diagnostics. The advantages of the private sector diagnostics are rapid, flexible approaches to project beyond that possible in a traditional diagnostic setting. Some disadvantages are a work environment more distant from academic resources and collegial interaction, the need to outsource services, and working without the operational infrastructure typically found in state and university laboratories.

Two keys to a successful career in poultry diagnostics are sound knowledge and skills in a diagnostic discipline, and the ability to merge this with an understanding of integrated poultry production. The core development of these future diagnosticians resides in student programs supported by the AAAP Foundation, support for graduate student research training, and postgraduate training programs and residencies, such as those recognized by the American College of Poultry Veterinarians.

The critical test for the future of poultry diagnostics is the addition of value to production efficiency, poultry welfare, and food safety. Within this framework, technology will continue to advance but the value will depend on successful application to mechanisms of disease and to efficient and humane poultry production. Quality system principles and practices will be integral to trustworthy communication and collaboration among diagnosticians and animal health regulators, and facilitation of domestic and global movement of healthy poultry and wholesome poultry products.

ACKNOWLEGEMENT

The author thanks John McCarty, Luis Gomez, Joan Schrader, Floyd Wilson, and Marty Hoerr for contributions to, and ongoing support of production-integrated pathology.

REFERENCES

Day J.M., B.B. Oakley, B. S. Seal, L. Zsak. Comparative analysis of the intestinal bacterial and RNA viral communities from sentinel birds placed on selected broiler chicken farms. PLoS ONE 10(1): e0117210. doi:10.1371/journal. pone.0117210. 2015.

NVSL History. https://www.aphis.usda.gov/publications/animal_health/content/printable_version/HelpingYouPoultryBreeder-PA1708-FinalJuly09.pdf retrieved January 16, 2017.

Oakley, B.B., C.A Morales, J. Line, M.E. Berrang, R.J. Meinersmann, G.E. Tillman, M.G. Wise, G.R. Sargusa, K.L. Liet, and B.S. Seal. The poultry-associated microbiome: network analysis and farm-to-

fork characterizations Published online doi: 0.1371/journal.pone. 0057190 PMCID: PMC3584146. Feb. 03, 2013.

Oakly, B.B., H.S. Lillehoh, M.H. Kogut, W.K. Kim, J.J. Mauer, A. Pedroso, M.D. Lee, S.R. Collett, T.J. Johnson, and N.A. Cox. The chicken gastrointestinal microbiome. FEMS Microbiology Letters 360:100-112. 2014.

Pantin-Jackwood. Multicausal enteric diseases. In Diseases of Poultry, 13th ed. Ed. D.E. Swayne, et al, Wiley-Blackwell. Pp. 1322-25, 2013.

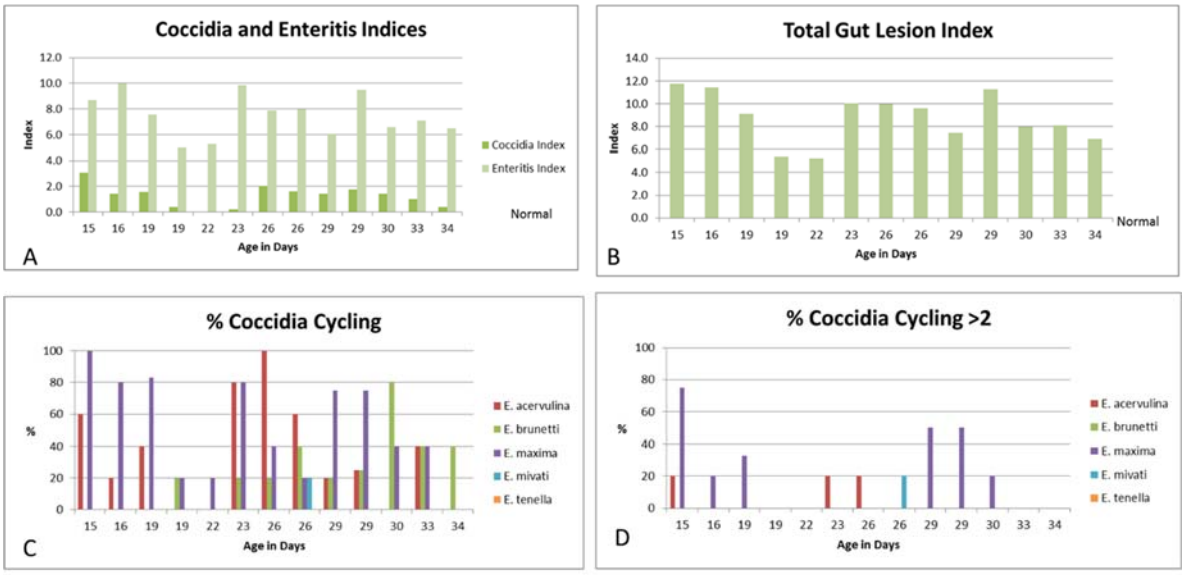
Roto, S.M., P.M. Rubinelli, S.C. Ricke, Review: An introduction to the avian gut microbiota and the effects of yeast based prebiotic-type compounds as potential feed additives. doi: 10.3389/fvets.2015.00028 PLoS One. 2013; 8(2): e57190. September 2015.

Shabbir, M.Z., T. Malys, Y.V. Ivanov, J. Park, M.A.B.Shabbir, M. Rabbbani, R. Yaqub, and E.T. Harvill, Microbial communities present in the lower respiratory tract of clinically healthy birds in Pakistan. Poult Sci 94 (4): 612-620. DOI: https://doi.org/10.3382/ps/pev010 . 2015.

University of Minnesota Diagnostic Laboratory History. http://www.vdl.umn.edu/about-us/our-history; retrieved January 16, 2017.

University of Wisconsin Diagnostic Laboratory History. http://www.wvdl.wisc.edu/index.php/about-wvdl/history-of-lab/; retrieved January 16, 2017.

Figure 3. Production-integrated pathology for a broiler program, with duodenum, jejunum, and cecum from clinically normal broilers examined histologically at 15 through 34 days of age. A) The Enteritis Index developed by semiquantitative lesion scoring, has an early peak 16 days, associated with replication of multiple species of *Eimeria*, but also lesions of viral enteritis (data not shown). Coccidia were observed from 15 through 34 days of age, the age limits of the survey. B) The Total Gut Lesion Index, representing the sum of the Enteritis and Coccidia Indices, shows an early peak at 15 to 16 days, and an extended second peak from 23 to 29 days, during which four species of *Eimeria* were observed. C) Multiple species of *Eimeria* were detected by histopathology. D) The most severe coccidia scores were associated with *E. maxima*, followed by *E. acervulina* and *E. mivati*.



USE OF A YEAST CELL WALL PRODUCT IN COMMERCIAL LAYER FEED TO REDUCE S. E. COLONIZATION

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SUMMARY

Salmonella Enteritidis (SE) is a concern from a human health perspective for cage layer hens due to its propensity to translocate to internal organs. It has been found that approximately one in 10,000 eggs can be SE infected (3). In this study, Hyline W-36 pullets fed a yeast cell wall product (YCW) from 10 weeks until study termination were then orally challenged with 3.0 x 10^9 cfu SE (at 16 weeks of age). The YCW product reduced SE prevalence in the ovaries and ceca. The YCW product significantly reduced SE counts (MPN method) in the ceca. SE can gain access to the egg by both transovarial route and egg shell contamination. The tested YCW product reduced SE in ovaries and significantly in the feces. Therefore, it can be a valuable tool in a SE reduction program for commercial layers.

INTRODUCTION

SE is a human foodborne illness concern with nearly 80% of cases between 1985 and 1999 attributed to table eggs (7). In challenge studies, it was found that approximately one in 10,000 eggs from SE infected hens contained SE (3). There are many methods commercial egg producers can employ to reduce SE introduction to their flocks. However, when these methods fail it is important to reduce both the opportunity for SE to colonize the layers' intestines and more importantly prevent egg transmission. One method used in broiler chickens to reduce intestinal *Salmonella* spp. colonization has been yeast cell wall components, often referred to as mannanoligosaccharides (MOS) (5,9). The mode of action of these MOS products is not fully understood. However, these polysaccharides are not digested by the birds' intestines and have been shown to agglutinate bacteria, affect the immune response, affect the microflora of the birds' intestines and enhance broiler and breeder performance (2,4,6,8). The β-Glucan portion of the yeast cell wall is very efficient at adhering *Salmonella* and therefore feeding a product higher in β-Glucan prior to and during the challenge of SE may be successful at reducing intestinal colonization and thus reducing the transovarial transmission of SE to the egg in commercial layer hens.

MATERIAL AND METHODS

Chickens. One-hundred ten-week of age commercial Hyline W-36 pullets were obtained from a commercial egg producer. At 12 weeks of age the pullet's day length was increased to 16 hours to stimulate ovarian development. The birds were housed in individual cages with 96 cages per treatment. Feed and water were *ad libitum* throughout the study.

Experimental design. At 10 weeks of age, upon arrival at test facility, all birds were started on test diets. Treatment 1 (T-1) had no additive and Treatment 2 (T-2) contained 0.5 ppm of the yeast cell wall (YCW) product IMW50 (Immuno Wall®), ICC, Sao Paulo, Brazil. When birds reached 16 weeks of age all were orally gavaged with 3.0 x 10^9 cfu/pullet nalidixic acid resistant SE. At 17 and 18 weeks of age (7 and 14 days post SE challenge) half of the hens at each time were humanely euthanized by cervical dislocation and ovaries and ceca aseptically removed, placed in sterile bags and weighed.

***Salmonella* Culture.** Sterile buffered peptone water was added to samples, serial dilution aliquot removed, then 10X tetrathionate was added in a 1:10 volume for prevalence. These samples were incubated overnight at 42°C and struck onto XLT-4 agar containing 25 µg/mL nalidixic acid. *Salmonella* enumeration utilized the micro most probable number (MPN) method of Berghaus et al. in 96 well two mL deep well blocks that contained 0.1 mL sample and 0.9 ml tetrathionate and 10 fold-dilutions performed (1). Blocks were incubated overnight at 42°C and then 1 µL of each well transferred to XLT-4 agar plates containing 25 µg/mL of nalidixic acid.

Statistical methods. *Salmonella* prevalences for ovary and ceca samples were compared between treatment groups by using Fisher's exact test. *Salmonella* MPNs were compared between treatment groups using the nonparametric Mann-Whitney test. For the comparison of *Salmonella* MPNs, samples with a negative culture result by the MPN method but a positive culture result by primary or secondary

enrichment were arbitrarily assigned an MPN value equal to one half the minimum detection limit of the MPN assay. MPN values were log-transformed prior to statistical analysis. All statistical testing assumed a two-sided alternative hypothesis, and $P < 0.05$ was considered significant. Analyses were performed using commercially available statistical software (Stata version 14.1, StataCorp LP, College Station, TX).

RESULTS AND DISCUSSION

Ovary results. At seven days post challenge, ovaries were tested for SE prevalence and number. At 14 days post challenge ovaries were tested for SE prevalence only. There were no statistically significant differences in ovary SE prevalence at both seven and 14 days. However, the IMW50 treated pullets had numerically lower prevalence with the seven day having the greatest difference (33% IMW50 to 41.7% nontreated) (Table 1). There was not a significant difference in MPN for those culture positive ovaries at seven days.

Ceca results. At both seven and 14 day collection, there was no significant difference in prevalence of SE in the ceca, Table 2. However, at both collection times, there was a consistent numerical reduction of SE positive ceca (93.8% IMW50 vs. 97.9% control at seven days and 47.9% IMW50 vs. 53.2% control at 14 days). In addition, at seven days the IMW50 S. E. positive hen ceca had significantly lower S.E. MPNs ($P = 0.016$) than those of the untreated group (Table 3). Since an extremely high SE challenge must be administered to result in ovary transmission, the challenge to the intestinal tract is extreme. Therefore, it is rare to observe significant prevalence differences in ceca. However, it is noteworthy that a consistent numerical reduction in the ceca was observed. More important was that positive ceca of IMW50-fed hens had significantly lower SE MPNs. This most likely resulted in the numerical reduction in prevalence observed in IMW50 hen ovaries versus control ovaries.

In summary, this study confirmed the work of Fernandez et al. that MOS products administered in feed can reduce ceca colonization of *Salmonella*. Further, this study demonstrated the effectiveness of these products when fed to 10 through 17 week old hens versus the Fernandez et al. study using broilers (2). Additionally, this study was able to demonstrate this reduction in ceca SE numbers results in reduced prevalence of SE in the critically important ovaries. Since SE can contaminate eggs by both transovarial route and fecal contamination on the egg shell, this study clearly indicated the MOS product, IMW50, high in β-Glucan can be effectively used in commercial layer pullets to reduce the SE risk in eggs by both routes of contamination.

REFERENCES

1. Berghaus, R. D., S. G. Thayer, B. F. Law, R. M. Mild, C. L. Hofacre, and R. S. Singer. Enumeration of Salmonella and Campylobacter spp. In environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. Appl. and Envir. Micro. 79:4106-4114. 2013.

2. Fernandez, F., M. Hinton, and B. VanGils. Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to Salmonella enteritidis colonization. Avian Path. 29:6, 575-581. 2000.

3. Gast, R. K. and C. W. Beard. Production of Salmonella enteritidis-contaminated eggs by experimentally infected hens. Avian Dis. 34:2, 438-446. 1990.

4. Kim, G.-B., Y. M. Seo, C. H. Kim, and I. K. Paik. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poultry Sci. 90:75-82. 2011.

5. Lourenco, M. C., L. N. Kuritza, R. M. Hayashi, L. B. Miglino, J.R. Duraw, L. Pickler, and E. Santin. Effect of a mannanoligosaccharide-supplemented diet on intestinal mucosa T lymphocyte populations in chickens challenged with Salmonella enteritidis. J. Appl. Poul. Res. 24:15-22. 2015.

6. Morales-López, R., E. Auclair, F. García, E. Esteve-Garcia, and J. Brufau. Use of yeast cell walls; β-1, 3/1, 6-glucans; and mannoproteins in broiler chicken diets. Poultry Sci. 88:601-607. 2009.

7. Patrick, Mary E., P. M. Adcock, T. M. Gomez, S. F. Altekruse, B. H. Holland, R. V. Tauxe, D. L. Swerdlow. Salmonella enteritidis infections, United States, 1985–1999. Emerg. Infect. Dis. 10:1-7. 2004.

8. Shashidhara, R. G. and G. Devegowda. Effect of dietary mannan oligosaccharide on broiler breeder production traits and immunity. Poultry Sci. 82:1319-1325. 2003.

9. Spring, P., Wenk, C., Dawson, K. A., and K. E. Newman. The effects of dietary mannanoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of Salmonella-challenged broiler chicks. Poultry Sci. 79:205-211. 2000.

Table 1. Ovary *Salmonella* prevalences in birds from each of two treatment groups by day post-challenge.

Day post-challenge	Treatment	n	No. positive (%)	$P^{\dagger}$
7	None	48	20 (41.7)[a]	0.527
	IMW50	48	16 (33.3)[a]	
14	None	48	2 (4.2)[a]	1.00
	IMW50	48	1 (2.1)[a]	

[†]Fisher's exact test. Within days, percentages with a superscript in common do no differ with a level of significance of 5%.

Table 2. Ceca *Salmonella* prevalences in birds from each of two treatment groups by day post-challenge.

Day post-challenge	Treatment	n	No. positive (%)	$P^{\dagger}$
7	None	48	47 (97.9)[a]	0.617
	IMW50	48	45 (93.8)[a]	
14	None	47	25 (53.2)[a]	0.683
	IMW50	48	23 (47.9)[a]	

[†]Fisher's exact test. Within days, percentages with a superscript in common do no differ with a level of significance of 5%.

Table 3. Summary of $\log_{10}$ *Salmonella* MPN per gram in culture-positive ceca samples from each of two treatment groups by day post-challenge.

Day post-challenge	Treatment	n	Mean (SD)	Median	$P^{\dagger}$
7	None	47	2.91 (1.35)	2.70[a]	0.016
	IMW50	45	2.30 (1.46)	2.23[b]	
14	None	25	0.40 (0.77)	0.07[a]	0.563
	IMW50	23	0.30 (0.61)	0.07[a]	

[†]Mann-Whitney test. Within days, medians with a superscript in common do no differ with a level of significance of 5%.

PREVENTING NECROTIC ENTERITIS WITHOUT ANTIBIOTICS

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SUMMARY

The poultry industry has significantly reduced the routine use of antibiotics either by regulations or by market requirements (No Antibiotics Ever, NAE programs). In the U.S. and Canada NAE programs also mean no use of ionophore anticoccidial drugs. Overall, the broiler disease necrotic enteritis (NE) is on an increase but especially in these NAE programs. *Eimeria maxima* has been shown to be one of the primary insults allowing *Clostridium perfringens* to proliferate and cause the disease NE in NAE programs. Results of three necrotic enteritis challenge studies using a *Bacillus licheniformis* and/or a *B. subtilis* with or without a natural anticoccidial product were evaluated. Data will be presented on the effectiveness of all products and combinations of these products to prevent not only the clinical disease necrotic enteritis, but more importantly, preventing subclinical effects of *C. perfringens* on body weight and feed efficiency of broilers.

INTRODUCTION

NE, in most instances, is a multifactorial disease that involves a coccidia infection of the small intestine and presence of a toxigenic strain of *Clostridium perfringens*. It has been demonstrated that once coccidia begin to create intestinal epithelium damage and the chicken's intestines responds with increased mucus production, *C. perfringens* counts can increase two-fold every 8-10 minutes (4). Preventative use of antibiotics in diets has been the most common means of preventing *C. perfringens* growth and subsequent toxin production. With the current regulatory and market climates for broiler chicken production away from the routine use of antibiotics, there has been increased research to find non antibiotic methods to either control the coccidia or *Clostridium* (12).

One of the most effective means of control of coccidia in poultry are ionophore anticoccidial drugs in the bird's diet (9). However, these compounds are the product of bacterial fermentation and therefore are considered an antibiotic in many countries. Thus ionophore anticoccidial drugs cannot be used in antibiotic free broiler production. There has been research into botanical alternatives for control of poultry coccidia. Many plant extracts have demonstrated both *in vitro* and *in vivo* effectiveness such as aloes, betaines, saponins, oregano, and others (1,2,3).

The use of probiotics to inhibit colonization of *Salmonella* in poultry has been known for many years and given the name competitive exclusion (10,11). Probiotics have also been demonstrated to inhibit growth of *C. perfringens* and reduce severity of the disease N.E. (6,8). *Bacillus* spp. that are spore forming probiotics called direct fed microbial (DFM) have the advantage of a heat tolerant spore that can survive gastic acid and become vegetative in the small intestine (5). *Bacillus subtilis* and *Bacillus licheniformis* are two commonly used DFM products. Both have demonstrated anti-clostridial activity *in vitro* and *in vivo* (7,13).

Since the disease necrotic enteritis exhibits in a clinical form (mortality) and subclinical form (lower body weight and poor feed efficiency), three floor pen studies were performed using a challenge model to produce both subclinical and clinical NE (16). The NE control was also directed toward control of *Clostridium* (*B. subtilis* and *B. licheniformis*) and *Eimeria* (Saponin product C.T.).

MATERIALS AND METHODS

In this study, a total of three *C. perfringens* challenge experiments were conducted in floor pens with space of 0.09 sq. m/bird (1.0 ft^2/bird). In all three studies, the antibiotic, bacitracin methylene disalicylate (BMD®), was a medicated control at 50 g/U.S. ton (55 g/metric ton).

Challenge model. The model used was to administer on the litter at 14 days 5000 oocysts *E. maxima* into each pen (6). Then on days 18, 19 and 20 of age a Net B and Alpha toxin positive strain of *C. perfringens* (CP#6) at approximately 1.0 x 10^8 cfu/bird in waterers. All mortality during the study was necropsied for cause of death. On day 21, three birds per pen were euthanized by cervical dislocation and middle of jejunum lesion scored for N.E., 0 = normal, 1 = mucus, 2 = classic necrotic enteritis, 3 = sloughed hemorrhagic mucosa (6).

Bird husbandry. Male by product of Ross 708 (Aviagen, Inc.) were obtained at one day of age and

were spray vaccinated prior to placement with a commercial coccidia vaccine (Advent®, Huvepharma, Inc.). Fifty birds per pen were placed onto reused litter from a previous N.E. study. Water and feed were provided *ad libitum*.

Statistical analysis. Means for cage weight gain, feed consumption, feed conversion (adjusted for mortality: feed consumed/final live weight + mortality weight), NE lesion scores, and NE mortality were calculated. The mortality was assessed by gross lesions on necropsy-enlarged dark-colored livers and the pseudomembrane appearance of classical NE in the small intestine. Statistical evaluation of the data was performed using a STATISTIX for Windows program (Analytical Software, Tallahassee, FL). The procedures used were general linear procedures using ANOVA with a comparison of means using least significant difference (*t*-test) (LSD (T)) at a significance level of 0.05.

RESULTS AND DISCUSSION

Study 1. Results for subclinical NE demonstrated DFM combined with the anticoccidial botanical (CT) and the chemical anticoccidial amprolium had the heaviest body weights and lowest FCR (Table 1). This was followed closely by DFM and the botanical alone. The lowest NE mortality was in the DFM alone, then the amprolium with the BMD having the same level of mortality as the untreated challenged at 2.8%. In this study, the challenge was considered mild as is often seen in U.S. broilers.

Study 2. The treatments containing the natural botanical with DFM then BMD followed by DFM alone had the numerically lowest FCR and also had improved body weight over the challenge control (Table 2). DFM alone or with the natural botanical (CT) had the lowest NE mortality and NE lesions. In this study, the NE challenge was more severe (20% in untreated control) which resulted in greater differences between treatments.

Study 3. As in study two, DFM with the natural botanical (CT) had the lowest FCR and heaviest body weight (Table 3). The challenge in this study was also severe with only the antibiotic BMD able to reduce mortality significantly from the challenge control.

In summary, these three studies confirmed that a DFM of *B. subtilis* and/or *B. licheniformis* can be as effective in reducing negative effects of *C. perfringens* on broilers growth rate and feed efficiency as the antibiotic BMD. This was observed consistently across three studies with mild to severe necrotic enteritis challenge. It was also demonstrated that combining DFM with a natural botanical can enhance the effect of DFM. This would indicate the anticoccidial effect of the botanical is effectively reducing damage by the *E. maxima* similar to the amprolium treatment (Table 1). This study confirms the work by Collier et al. that the coccidia damage of the epithelium and this resulted in the rapid proliferation of *C. perfringens* (4). These three studies demonstrate a combination of DFM and the botanical anticoccidial product can be as effective as the antibiotic, BMD, in preventing both clinical and subclinical N.E.

REFERENCES

1. Abbas, R. Z., D. D. Colwell, and J. Gilleard. Botanicals: an alternative approach for the control of avian coccidiosis. World's Poultry Sci. 68:203-254. 2012.

2. Alfaro, D. M. A., V. F. Silva, S. A. Borges, F. A. Maiorka, S. Vargas, and E. Santin. Use of *Yucca schidigera* extract in broiler diets and its effects on performance results obtained with different coccidiosis control methods. Res. 16:248-254. 2007.

3. Bozkurt, M., I. Giannenas, K. Kucukyilmaz, E. Christaki, and P. Florou-Paneri. An update on approaches to controlling coccidia in poultry using botanical extracts. Br. Poultry Sci. 54, 6, 713-727. 2013.

4. Collier, C. T., C. L. Hofacre, A. M. Payne, D. B. Anderson, P. Kaiser, R. I. Mackie, and H. R. Gaskins. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet. Immuno. and Immunopath. 122:104-115. 2008.

5. Cutting, S. Review Bacillus probiotics. Food Microbiol. 28:214-220. 2011.

6. Hofacre, C. L., R. Froyman, B. Gautrias, B. George, M. A. Goodwin and J. Brown. Use of Aviguard and other intestinal bioproducts in experimental *Clostridium perfringens*-associated necrotizing enteritis in broiler chickens. Avian Dis. 42:579-584. 1998.

7. Knap, I., B. Lund, A. B. Kehlet, C. Hofacre and G. Mathis. *Bacillus licheniformis* prevents necrotic enteritis in broiler chickens. Avian Dis. 54:931-935. 2010.

8. Kaldhusdal, M., C. Schneitz, M. Hofshagen and E. Skjerve. Reduced incidence of *Clostridium perfringens*-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. Avian Dis. 45:149-156. 2001.

9. McDougald, L. R. and S. H. Fitz-Coy. Coccidiosis. In: Diseases of Poultry, 13th ed. Wiley-Blackwell. D. Swayne, editor. Pp. 1148-1163. 2013.

10. Mead, G. C. Prospects for 'competitive exclusion' treatment to control salmonellas and other

foodborne pathogens in poultry. The Vet. J. 159:111-23. 2000.

11. Rantala, M. and E. Nurmi. Prevention of the growth of Salmonella infantis in chicks by the flora of the alimentary tract of chickens. British Poultry Science 14:627-630.1973.

12. Smith, J. A. Experiences with drug-free broiler production. Poultry Sci. 90:2670-2678. 2011.

13. Teo, A. Y-L., and H.-M. Tan. Inhibition of *Clostridium perfringens* by a novel strain of *Bacillus subtilis* isolated from the gastrointestinal tracts of healthy chickens. Appl. & Environ. Micro. 71:4185-4190. 2005.

Figure 1.

	Treatments									
I.D.	Study 1			**I.D.**	Study 2			I.D.	Study 3		
	Treatment Description				Treatment Description				Treatment Description		
	Starter (D0-14)	Grower (D15-35)	Finisher (D35-42)		Starter (D0-14)	Grower (D15-35)	Finisher (D35-42)		Starter (D0-14)	Grower (D15-35)	Finisher (D35-42)
T1	-BMD* 50g/ton	-BMD 50g/ton	-BMD* 50g/ton	T1	No additive	No additive	No additive	T1	No additive	No additive	No additive
T2	-OBL** 0.5 lb/ton -OBS*** 1 lb/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton	T2	No additive	No additive	No additive	T2	No additive	No additive	No additive
T3	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton	T3	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton	T3	CT 90 g/ton	CT 90 g/ton	CT 90 g/ton
T4	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT[1] 90 g/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton Amprolium* 113g/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton -CT 90 g/ton	T4	-OBL 0.5 lb/ton -OBS 1 lb/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton	-OBL 0.5 lb/ton -OBS 1 lb/ton	T4	OBL 1 lb/ton	OBL 1 lb/ton	OBL 1 lb/ton
T5	No additive	Amprolium* 113g/ton	No additive	T5	-CT 90 g/ton	-CT 90 g/ton	-CT 90 g/ton	T5	OBL 1 lb/ton CT 90 g/ton	OBL 1 lb/ton CT 90 g/ton	OBL 1 lb/ton CT 90 g/ton
T6	No additive	No additive	No additive	T6	-BMD 50g/ton	-BMD 50g/ton	-BMD 50g/ton	T6	BMD 50g/ton	BMD 50g/ton	BMD 50g/ton

*BMD = Bacitracin methylene disalicylate
**OBL = Bacillus licheniformis
***OBS = Bacillus subtilis
[1] CT = Botanical anticoccidial

Table1.

Table 1. Study 1 Clinical and Subclinical Necrotic enteritis*

	Treatment	Feed Conversion 0 – 42 day	Weight gain 0 – 42 day	Lesion Score Day 22	Mortality % N.E.
T1	BMD	1.622^{abc}	2.206^{c}	0.50^{a}	2.8^{a}
T2	OBL/OBS	1.628^{ab}	2.298^{b}	0.50^{a}	0.3^{b}
T3	OBL/OBS/CT	1.593^{bc}	2.292^{b}	0.33^{a}	2.5^{a}
T4	OBL/OBS/CT Amprol Grower	1.584^{c}	2.382^{a}	0.42^{a}	2.3^{ab}
T5	Amprol Grower	1.633^{a}	2.234^{bc}	0.29^{a}	1.8^{ab}
T6	No Additive Challenged	1.630^{ab}	2.226^{bc}	0.38^{a}	2.8^{a}

*Means in columns with no common letter (a,b,c,d) are significantly different (P < 0.05).

Table 2.

Table 2. Study 2 Clinical and Subclinical Necrotic enteritis*					
	Treatment	Feed Conversion 0 – 42 day	Weight gain 0 – 42 day	Lesion Score Day 22	Mortality % N.E.
T1	No Additive/ No Challenge	1.760[b]	2.116[a]	0.08[d]	0.0[c]
T2	No Additive/ Challenge	1.841[a]	2.074[a]	0.96[a]	20.0[a]
T3	OBL/OBS/CT	1.733[b]	2.141[a]	0.58[c]	13.5[ab]
T4	OBL/OBS	1.741[b]	2.130[a]	0.67[bc]	9.0[b]
T5	CT	1.750[b]	2.153[a]	0.88[ab]	12.5[b]
T6	BMD	1.736[b]	2.146[a]	0.71[abc]	14.0[ab]
*Means in columns with no common letter (a,b,c,d) are significantly different (P < 0.05).					

Table 3.

Table 3. Study 3 Clinical and Subclinical Necrotic enteritis*					
	Treatment	Feed Conversion 0 – 42 day	Weight gain 0 – 42 day	Lesion Score Day 22	Mortality % N.E.
T1	No Additive/ No Challenge	1.710[ab]	2.333[a]	0.05[a]	1.1[b]
T2	No Additive/ Challenge	1.715[a]	2.273[ab]	0.40[a]	20.0[a]
T3	CT	1.735[a]	2.188[b]	0.33[a]	26.1[a]
T4	OBL	1.711[ab]	2.295[ab]	0.43[a]	16.7[a]
T5	OBL/CT	1.675[b]	2.358[a]	0.30[a]	23.9[a]
T6	BMD	1.734[a]	2.234[ab]	0.28[ab]	13.6[ab]
*Means in columns with no common letter (a,b,c,d) are significantly different (P < 0.05).					

A CASE OF LISTERIOSIS IN BACKYARD CHICKENS

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INTRODUCTION

Listeria monocytogenes has a worldwide distribution, and has been estimated to cause approximately 1,500 hospitalizations and 250 deaths in the United States each year (1). *L. monocytogenes* can be identified in soil, water silage and animal feces (2). Outbreaks of Listeriosis, caused by *L. monocytogenes* occur sporadically in chickens, waterfowl, pigeons, turkeys and other avian species (3); with young birds being the most susceptible(4). The organism is important because of its ability to cause human infections following contact with infected birds (3) or consumption of contaminated poultry or poultry products, especially those that are precooked and "ready to eat (5)."

CASE HISTORY

Two 12-week-old cockerels were submitted to the Indiana Animal Disease Diagnostic Laboratory on September, 21, 2016 for necropsy. The owner noticed a 30% increase in mortality in the flock and reported that young birds were becoming sick and dying within 24 hours. Other birds in the flock were inappetent, displayed neurological clinical signs (wry neck and leg paralysis) and were extremely weak and lethargic.

Gross Necropsy, Histopathology and Ancillary Testing. Gross necropsy revealed:

1) Intestines—enteritis with petechial hemorrhage and intralesional coccidial oocyts, as well as intraluminal nematodes. Nematodes were later identified as *Heterakis* spp. via fecal floatation.

2) Heart—Epicarditis, myocarditis and hydropericardium.

3) Whole body—generalized emaciation and, 4) Spleen—splenomegaly. Histopathology revealed: 1) Kidney, liver, air sac, lung, heart, sciatic and brachial nerves, and intestines—lymphosarcoma; 2) Intestines—coccidial enteritis with intraluminal nematodes; 3) Ventriculus—necrotizing ventriculitis

4) Pancreas—necrotizing pancreatitis.

5) Heart—myocardial necrosis. Lastly, *L. monocytogenes* was cultured aerobically from the heart and a liver/spleen pool.

Diagnoses. 1) Listeriosis, 2) Marek's disease, and 3) coccidiosis.

DISCUSSION

Listeriosis is common in cooler, wet environments like those associated with flooding. It is important for prevention purposes to limit flooding around the coop as much as possible. It is also important to note that *Listeria monocytogenes* is a zoonotic disease and strict care should be taken when handling birds, especially any showing clinical signs. It is recommended that birds showing clinical illness not be consumed due to the presence of *Listeria monocytogenes* on the premise. Increased moisture in the environment, as seen with flooding is also conducive to sporulation of coccidial oocysts. Coccidiosis is exacerbated by any acute or chronic illness that decreases the immunocompetence of the bird. Marek's disease is a common lymphoproliferative disease of chickens (2) and likely attributed to the neurological clinical signs, and increased morbidity and mortality in the flock.

REFERENCES

1. Scallan, E., R.M Hoekstra, F.J. Angulo, R. V. Tauxe, M. A. Widdowson, S.L. Roy, J. L. Jones, and P. M. Griffin 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis.* 17: 7-15.

2. Swayne, D., Glisson, J., McDougald, L., Nolan, L., Suarez, D., & Nair, V. (2013). *Diseases of Poultry* (13th ed.). Hoboken: Wiley.

3. Gray, M. L. and A. H. Killinger. 1966. *Listeria* monocytogenes and liseric infections. *Bacteriol Rev.* 30: 309-382.

4. Basher, H.A., Fowler, D.R., Rodgers, F.G., Seaman, A., & Woodbine, M. (1984). Pathogenicity of natural and experimental listeriosis in newly hatched chicks. *Research in Veterinary Science, 36*(1), 76-80.

5. Cox, N.A., J.S. Bailey, E. T. Ryser, and E. H. Marth.1999. Incidence and behavior of *Listeria monocytogenes* in poultry and egg products. *In: Listeria, Listeriosis and Food Safety*, 2nd ed. Ryser, E. T., and E. H. Marth, eds. Marcel Dekker, Inc., New York. 565-600.

EFFECT OF THE MICROBIAL PRODUCT GALLIPRO® HATCH *IN OVO* INOCULATION ON YOUNG CHICK VIABILITY AND *ENTEROCOCCUS FAECIUM* M74 IN GUT AND CECA

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INTRODUCTION

At birth, animals receive a natural inoculation of microbes, which establish themselves into the intestine. The colonization of beneficial bacteria allows the animal to resist potential environmental challenges. In birds, this inoculation happens in the nest through contact with the hen and nest materials. Modern poultry production excludes the contact between chick and the hen, and depends mostly on bacteria present in the hatchery and barn environment.

Artificial incubation in poultry also delays enteric tract colonization by desirable microorganisms due to lack of contact with adult birds. Unfortunately, chicks can be exposed to pathogens while still at the hatchery, during hatching, sexing, vaccination, and transport, even before they consume their first feed. Initial microbe colonization is not only important to prevent pathogenic bacteria to colonize by competitive exclusion (1), but it is also very important to stimulate the development and maturation of the immune system and therefore to promote health (2).

The objective of this study was to determine the impact of GalliPro® Hatch (GPH, *Enterococcus faecium* M74, 1.4 X 10^7 CFU/egg or 50 µL/egg) using Inovoject® technology on hatchability when administered *in ovo* and on the viability of chicks during the first week of life. The second objective was to evaluate the occurrence of *Enterococcus faecium* M74 in gut and ceca.

MATERIALS AND METHODS

Animals and experimental design. The study was run at the Poultry Science Department of the Mississippi State University, in Jan-Mar 2016. Three treatments were: negative control, Marek's disease vaccine (MDV) and MDV + GPH). Table 1 shows the experimental design of the trial.

A total of 3,906 Ross-708 fertilized eggs were purchased from a commercial breeder farm where the breeder flock was at least 55 weeks of age. All eggs were weighed and the average egg weight identified. All eggs were sorted by weight, assigned to a treatment and placed into either a Jamesway PS500 incubator. A total of 684 day old chicks were used for post hatch first week performance evaluation.

On day 10, eggs were candled. Infertile, on day 18, labelled eggs were commercially injected with Marek's vaccine or a combination of Marek's vaccine and GalliPro Hatch, on day 21 all hatched chicks were counted. Eggs that do not hatch were broken out for hatch residue.

Samples and Sampling Day (Hatchery):

Day 0: Sorting by weight, treatment allocation, and setting

Day10: Fertility (candling)

Day 18: *in ovo* application

Day 21: Hatch residue, chick weights, and Feather sexing

There were a total of 91 egg flats used to incubate 3842 eggs. Each flat holds 42 eggs which is the required number of eggs for the commercial *in ovo* equipment being used for injection. All egg flats were randomly assigned positions within the incubator through day 18 when actual treatments were applied. At that time eggs were moved into hatching baskets and set into the hatcher. There were six compartments within each hatching basket and each compartment accommodated a total of 21 eggs, this then required two compartments for each flat of eggs.

Once all eggs had their appropriate *in ovo* treatment applied, they were placed into the hatcher until hatch. To reduce cross contamination between treatments, the eggs receiving the lowest treatment dose (Treatments 1 and 2) were placed on top of the hatcher with the Treatment 3 (GPH) at the bottom.

***E. faecium* count and identification**. As individual chicks were counted they were feather sexed and all male chicks were weighed and moved into a grow-out facility. On day one (actually 12 hours after hatch) and day seven, five birds from Treatment 1 and 10 birds from Treatments 5 and 6 were culled using individual pens. Each bird was humanely euthanized and the yolk sack (YS), cecal tonsils (CT), and remaining intestinal tract (IT) were placed in sterile collection tubes using sterile laboratory techniques. The collection tubes were snap frozen in

either (liquid nitrogen or dry ice) and stored in a -80°C freezer until shipped to Chr. Hansen laboratory in Denmark for *E. faecium* counts and identification. Three samples from YS, CT, and IT from one-day-old and seven-day-old chickens were recovered for analysis. Counting of CFUs was undertaken using TSA blood agar and Enterococcus selective agar. One randomly collected colony from each sample of YS, CT and IT was cultivated to ensure purity in preparation for PFGE typing. A M74 reference strain and the collected colonies from the intestinal sample cultures were typed by Pulsed Field Gel Electrophoresis (PFGE).

The data were analyzed with SAS (SAS Institute Inc., Cary, NC) for randomized complete block design. Egg flat was the experimental unit. Significance was accepted at $P \leq 0.05$ using Fisher's Protected LSD.

RESULTS AND DISCUSSION

***In ovo* results**. The results of this research indicate that injecting 18-day-old embryos with Marek's vaccine or a combination of Marek's vaccine and GalliPro Hatch did not impact any variable except late dead embryos. An issue with temperature distribution throughout the hatcher occurred, due to a higher percentage of late dead embryos for all treatments when compared to industry standards. Even though an issue occurred in the hatcher the results clearly demonstrate that any puncture of the egg shell, impacts the percentage of late dead embryos. The fact that 1.4×10^7 CFU of bacteria can be introduced to an 18-day-old embryo and not cause contamination or impact overall hatchability or average chick weight is very positive. Especially when trying to establish a method for seeding the gastro-intestinal microbiome which could impact performance and health throughout the entire life of the broiler chicken. Treatment 1 does not reflect the reality in the poultry industry because Marek's vaccine is routinely applied. When comparing Treatment 2 with Treatment 3, it is important to highlight the fact that GalliPro Hatch inoculation did not impair hatchability at all. Actually, GalliPro Hatch (Treatment 1) even reduced numerically the late dead compared to Treatment 3 and 4 groups. Hatchability results are shown in Table 2.

First week of life (0-7 days). The main effects from the incomplete factorial analysis also provided no difference for live weight gain per bird, end weight per bird, feed intake per bird, percent mortality, or feed conversion ratio for the first seven day period.

Overall the live performance data suggests that Marek's vaccine and GalliPro Hatch *in ovo* application does not negatively impact on live performance.

The main objective of the study was to see the effect of inoculating GalliPro Hatch on the survival of birds during the first week of life. Although there was no significant difference between these three treatments during the first week of life, it has to be noted that birds from eggs inoculated with GalliPro Hatch showed 50% less mortality than birds inoculated only with the vaccine (1.316% vs 2.632%). This important numerical difference might become significant under the commercial environment or high stress condition. Table 3 shows the performance result among Treatments 1, 2 and 3 during the first seven days of life.

The *E. faecium* CFU counts for chicks receiving GalliPro Hatch *in ovo* are shown in Table 4. In general, a high number of uniform bacterial cultures were discovered. When visually inspecting the colonies, many looked homologous and showed the typical colony morphology of an *Enterococcus*. The identification of *Enterococcus faecium* (M74) was confirmed by DNA fingerprinting (PFGE). The presence of the probiotic strain in the gut is a critical measurement to prove that the inoculated bacteria are viable in the intestinal tract. Results showed that *Enterococcus faecium* (M74) from the product GalliPro Hatch was present in high concentration in the yolk sac, cecal and intestinal samples of one-day-old and seven-day-old chickens. Results demonstrate that the inoculated strain (M74) is absolutely viable for intestinal colonization through *in ovo* injection.

CONCLUSION

The chicks' tissue sample results demonstrate that the inoculated strain (M74) is viable for intestinal colonization through *in ovo* injection. The hatchery study indicated that GalliPro Hatch can be introduced by *in ovo* to an 18 day old embryo with no negative impact to hatchability characteristics (3). The days zero through seven live performance data suggest that injecting an embryo with Marek's vaccine and GalliPro Hatch has no negative effect on live performance. Although there was no significant difference during the first week of life, it has to be noted that birds from eggs inoculated with GalliPro Hatch showed 50% less mortality compared to the birds inoculated only with the vaccine (1.316% vs 2.632%). This numerical difference can significant under more stressful, commercial conditions, as well as under the antibiotic free hatchery facility.

REFERENCE

J. E. de Oliveira, E. van der Hoeven-Hangoor, I. B. van de Linde, R. C. Montijn, and J. M. B. M. van der Vossen. In ovo inoculation of chicken embryos

with probiotic bacteria and its effect on posthatch Salmonella susceptibility. 2014, Poultry Science 93: 818-829

Chasity M. Pender, Sungwon Kim, Tiffany D. Potter, Miranda M. Ritzi, Mark Young, and Rami A. Dalloul. In ovo supplementation of probiotics and its effects on performance and immune-related gene expression in broiler chicks. 2016 Poultry Science 10: 3382

C. Beck, P.H. Mota, C.D. McDaniel, E.D. Peebles, K.G.S. Wamsley, A., Blanch, M. Rouault, and A.S. Kiess. Injecting Gallipro® hatch into fertile broiler hatching eggs using commercial *in ovo* technology and its impact on hatchability. 2016 Poultry Science annual meeting Abstract.

Table 1. Experimental treatments.

In-ovo Treatment	Treatment 1*	Treatment 2	Treatment 3
MDV	-	+	+
GPH, 1.4E+07 CFU/egg	-	-	+
Eggs/Flat	42	42	42
# of Flats	31	31	31
Eggs/Trt	1302	1302	1302
Post Hatching			
Birds/Pen	19	19	19
Pens/Trt	12	12	12
Birds/Trt	228	228	228

*No punch/injection (Negative control)

Table 2. Hatchery results.

Variable	Treatment 1	Treatment 2	Treatment 3	SEM	P value
Egg Set Weight	69.2	69.1	69.3	0.0002	0.69
Avg. Chick Weight	49.1	48.7	49.1	0.22	0.33
Hatchability, %	86.09	80.5	80.8	2.17	0.13
Infertile, %	0.00	0.08	0.00	0.04	0.37
Early Dead, %	0.31	0.23	0.22	0.15	0.91
Mid Dead, %	0.31	0.61	0.52	0.19	0.52
Late Dead, %	8.91[a]	14.52[b]	12.62[b]	0.18	0.019
Pipped, %	1.31	1.54	1.56	0.40	0.88
Cracked, %	0.08	0.08	0.00	0.06	0.60
Contaminated, %	0.23	0.08	0.22	0.11	0.56

[ab] Figures in the same raw with different superscript letters are significantly different ($p<0.05$)

Table 3. Effect of *in ovo* treatment on broiler performance during first 7-day period.

Variable	Treatment 1	Treatment 2	Treatment 3	SEM	P value
Live Wt gain/Bird	0.121	0.124	0.121	0.005	0.83
End Wt/Bird	0.17	0.173	0.169	0.005	0.83
Feed Intake/Bird	0.155	0.159	0.149	0.004	0.26
Mortality %	0.877	2.632	1.316	0.790	0.28
Feed Conversion Ratio	1.289	1.292	1.253	0.042	0.78

Table 4. *E. faecium* counts of the birds receiving GalliPro® Hatch *in ovo* (Treatment 5).

Age of birds	YS (CFU/g)	CT (CFU/g)	IT (CFU/g)
Day 1 (n = 3)	3.8E+09	2.4E+08	1.6E+07
Day 7 (n = 3)	5.0E+08	4.7E+06	2.8E+05

EFFECT OF HN PROTEIN LENGTH TO NEWCASTLE DISEASE VIRUS VIRULENCE, REPLICATION, AND BIOLOGICAL ACTIVITIES

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SUMMARY

The hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) plays important roles in viral invasion and maturation, with three distinct activities: receptor binding, neuraminidase (NA) activity, and fusion promotion. It was found that the HN proteins vary in length, and at least nine different length variants have been reported to date. To evaluate the effect of HN protein length to NDV virulence, replication and biological activities, a series of recombinant NDVs containing truncated or extended HN proteins based on an infectious clone of genotype VII NDV (SG10 strain) were generated. The mean death times and intracerebral pathogenicity indices of these viruses showed that the different length mutations in the HN protein did not alter the virulence of NDV. In vitro studies of recombinant NDVs containing truncated or extended HN proteins revealed that the extension of HN protein increased its hemadsorption (HAd) ability and impaired its neuraminidase activity, fusogenic activity and replication. Our results demonstrate that the HN biological activities affected by the C-terminal extension are associated with NDV replication but not its virulence. These findings may also be applicable to other paramyxoviruses.

INTRODUCTION

NDV is a member of the genus Avulavirus within the family Paramyxoviridae. It has a nonsegmented, negative-sense single-stranded RNA genome, which is approximately 15 kb long and encodes six major structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), HN, and large polymerase protein (L) respectively. HN is a multifunctional molecule that is responsible for the attachment of the virus to its sialic-acid-containing receptors, and has NA activity to hydrolyze the sialic acid molecules from progeny viral particles, and promotes membrane fusion through its interaction with F protein (4).

It was found that the HN proteins vary in length: 570 amino acids (570aa), 571aa, 572aa, 577aa, 578aa, 580aa, 582aa, 585aa, and 616aa (1). Previous study on the basis of an infectious clone of the lentogenic vaccine virus Clone-30 has shown that the pathogenicity of NDV is not influenced by the length variations in the 571aa, 577aa and 616aa HN proteins (5). But in the context of the virulent strains or other length variants, especially the unnatural length of HN protein, the data are lacking. Furthermore, these length differences arise from changes in the length of the C-terminal global head, the main functional region of HN, and thus they may influence the activity of the HN protein, and even the biological characteristics of the virus.

In this study, we chose a virulent isolate of NDV genotype VIId circulating in China (SG10) to build the recombinant NDVs encoding truncated or extended HN proteins. We used these to evaluate the role of HN protein length in the NDV virulence, replication and biological activities.

MATERIALS AND METHODS

Virus rescue. Virus rescue was performed as described previously (3).

Virulence of recovered viruses. The virulence of recombinant viruses was determined with standard virulence tests for NDV: MDT and ICPI.

Viral growth characteristics. The growth characteristics of the viruses were evaluated from their multicycle growth kinetics in a chicken embryo fibroblast cell line (DF-1) as described previously (3).

HAd, NA, and fusion index assays. The HAd activity, NA activity and fusion index were assayed as described previously (2).

RESULTS

Recovery of recombinant viruses. The full-length plasmids encoding 10 different length variants of the HN protein were constructed to rescue recombinant viruses. Eight recombinant viruses were rescued, but rNDV-SG10-HN565 and rNDV-SG10-HN566 were not (Fig. 1A).

Virulence of the recombinant viruses. The MDT values for all eight rescued viruses ranged from

39.0 to 43.5 h, which were very similar to the wild-type NDV-SG10-HN571 value (42 hours), and the results of the ICPI test were consistent with the results of the MDT. The ICPI values for all eight rescued viruses and NDV-SG10-HN571 were > 1.90. Based on both the MDT and ICPI values, all these viruses were classified as velogenic strains. These results indicate that the length of the HN protein has no obvious effect on NDV pathogenicity.

Growth properties of the mutant viruses. The growth characteristics of the viruses were evaluated from their multicycle growth kinetics in DF-1 cells (Fig. 1B). The results showed that the replication kinetics of all the HN-truncated mutant viruses were similar to those of wild-type NDV-SG10-HN571 and rNDV-SG10-HN571, whereas rNDV-SG10-HN582 and rNDV-SG10-HN616 showed delayed growth and significantly lower viral yields than the wild-type and parental recombinant viruses at 24 hpi. These results indicate that the HN protein truncation mutations negligibly influenced the replication efficiency of NDV in cells, but that the extension mutations substantially reduced the efficiency of NDV replication.

HAd, NA, and fusogenic activities of the recombinant viruses. Five representative HN length mutant viruses were assessed for their HAd, NA and fusogenic activities. The HAd activity of rNDV-SG10-HN582 was 134% of that of rNDV-SG10-HN571, which was the greatest increase in all the mutants, followed by 120% for rNDV-SG10-HN616. The HAd activities of rNDV-SG10-HN567 and rNDV-SG10-HN577 did not differ significantly from those of rNDV-SG10-HN571 (Fig. 1C). All the extended HN length mutant viruses showed significantly reduced NA activity relative to that of the parental recombinant virus, with the following values (relative to rNDV-SG10-HN571, 100%): rNDV-SG10-HN577, 89%; rNDV-SG10-HN582, 78%; and rNDV-SG10-HN616, 64% (Fig. 1D). These results suggested that the extension of HN protein increased the hemadsorption capacity, but reduced the NA activity. Quantification of the fusogenic abilities of the mutants (fusion index compared with the parental level) showed that rNDV-SG10-HN577, rNDV-SG10-HN582, and rNDV-SG10-HN616 had significantly reduced fusogenic activity (89%, 58%, and 77%) (Fig. 1E). These results indicate that the extension of the HN protein seriously impairs the fusogenic ability, whereas truncation mutations of the HN protein have no statistically significant influence on the fusogenic capacity.

DISCUSSION

In the present study, a genotype VIId NDV strain (SG10) with a 571aa HN protein was used as the model virus to construct the recombinant NDVs encoding truncated or extended HN proteins. The MDT and ICPI results indicated that mutations affecting the length of HN did not alter the virulence of the mutant viruses. These results are consistent with a previous report of Clone-30 NDV strain (5). This suggests that NDV pathogenicity is not influenced by the length of the HN protein, in either velogenic or lentogenic strains. The 570aa HN protein is the shortest length detected, as previously reported. In this study, rNDV-SG10-HN567 was successfully recovered. rNDV-SG10-HN565 was not recovered and a sequence revertant of rNDV-SG10-HN566 was recovered after passage in embryonated SPF chicken eggs. These results indicate that the length of the HN protein cannot be reduced indefinitely, and we suppose that this may relate to the evolution of NDV.

Our in vitro studies of NDV support the hypothesis that the length of the HN protein affects some biological functions of the virus. Extension of the HN protein increased NDV HAd activity, reduced its NA activity and fusion index. These results emphasize the importance of the 571aa HN protein in the NA and fusogenic activities of NDV strain SG10. The impairment of the NA and fusogenic activities induced by the extension of the HN protein is consistent with the viral replication observed in cells. However, the HAd activity of HN appeared to be independent of its NA and fusogenic activities. Our results suggest that these altered activities could affect viral growth in vitro and the extension of HN allows viral attachment but prevents virion release during the life cycle of NDV.

REFERENCES

1. Gould, A.R., E. Hansson, K. Selleck, J.A. Kattenbelt, M. Mackenzie, and A.J. Della-Porta. Newcastle disease virus fusion and haemagglutinin-neuraminidase gene motifs as markers for viral lineage. Avian Pathol. 32:361-373. 2003.

2. Huang, Z., A. Panda, S. Elankumaran, D. Govindarajan, D.D. Rockemann, and S.K. Samal. The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. J. Virol. 78:4176-4184. 2004.

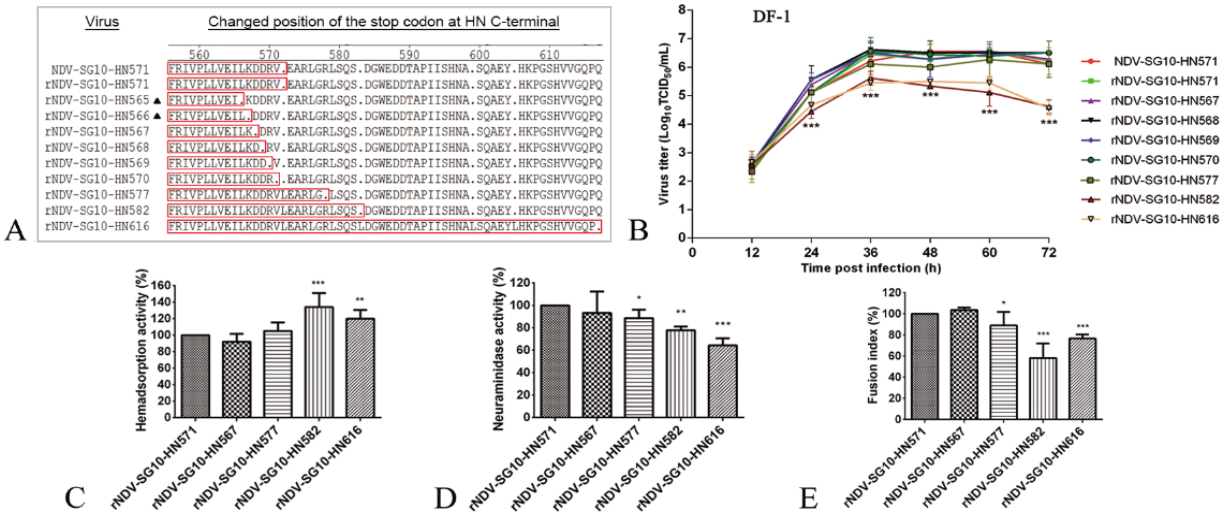
3. Liu, M.M., J.L. Cheng, X.H. Yu, Z.M. Qin, F.L. Tian, and G.Z. Zhang. Generation by reverse genetics of an effective attenuated Newcastle disease virus vaccine based on a prevalent highly virulent Chinese strain. Biotechnol Lett. 37:1287-1296. 2015.

4. Porotto, M., Z. Salah, I. DeVito, A. Talekar, S.G. Palmer, R. Xu, I.A. Wilson, and A. Moscona. The second receptor binding site of the globular head of the Newcastle disease virus hemagglutinin-neuraminidase activates the stalk of multiple paramyxovirus receptor binding proteins to trigger fusion. J. Virol. 86:5730-5741. 2012.

5. Romer-Oberdorfer, A., O. Werner, J. Veits, T. Mebatsion, and T.C. Mettenleiter. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. J. Gen. Virol. 84:3121-3129. 2003.

(The full-length article of Jihui Jin'manuscript has been published in Scitific reports.)

Figure 1. Recovery, growth, and biological activities of recombinant NDVs



DEVELOPMENT OF DIAGNOSTIC MULTIPLEX REAL-TIME PCRS FOR THE DETECTION OF *MYCOPLASMA GALLISEPTICUM*, *MYCOPLASMA SYNOVIAE*, AND INFECTIOUS LARYNGOTRACHEITIS IN CHICKENS

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SUMMARY

Avian respiratory diseases in poultry, resulting from *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), and infectious laryngotracheitis virus (ILTv) infection, are responsible for severe economic losses worldwide. Early detection is a key component of a control program and real time PCR can be an important tool in this respect. In this study, duplex real-time PCRs were developed and validated for simultaneous detection of MS or MG and ILT. The novel duplex real-time PCRs were both sensitive and specific for their intended targets.

INTRODUCTION

MS, MG, avian influenza (AI), and infectious laryngotracheitis (ILT) are economically significant respiratory pathogens affecting chickens. There is increasing need for rapid detection of all relevant respiratory pathogens in order to properly diagnose flocks suspected of respiratory disease. In the preliminary steps to creating a novel respiratory panel for detection of these pathogens, we developed duplex real-time PCRs for simultaneous detection of MG and ILT as well as MS and ILT. The duplex PCRs target previously described and widely used genes, MG hypothetical lipoprotein (MGA_0319), MS 16S-23S rDNA ISR, and ILT glycoprotein C (gC). The multiplex PCRs were validated against previously established individual real-time PCR methods using tracheal swabs from broilers experimentally infected with MS, MG, and ILT and plasmids containing the genes of interest. These tests are expected to lower costs and shorten wait time for diagnostic PCR results.

MATERIALS AND METHODS

Tracheal samples. Three-week-old broilers were inoculated with 100µL each of MG R-strain (n=43) via aerosol spray and MS strain K6677 (n=43) via eyedrop and footpad injection (50 µL each). Five and six days post MS and MG inoculation, respectively, the birds were vaccinated with a 2X dose (log106.32 TCID50/mL) of Laryngo-Vac® ILT vaccine (Zoetis, Florham Park, NJ). At four, seven, and 14 days post ILT vaccination, tracheal swabs were collected from each bird. All swabs were stored at 80°C until extraction. Each sample (n=245) was extracted using the Omega Mag-bind Viral DNA/RNA extraction kit on the ABI MagMAXTM Express-96 Deep Well Magnetic Particle Processor following the manufacturer's instructions and DNA was eluted into 50µL H2O.

Plasmids. Target genes for MGA_0319, MS 16S-23S ISR, and ILT gC (GeneBank Accession #s: NC_004829 (MG), AY768810 (MS), and NC_006623 (ILT)) were amplified using previously developed primers and probes (1, 2, 3) and cloned into plasmids using the TOPO® TA Cloning® Kit for Sequencing with OneShot® TOP10 Chemically Competent E. coli (Life Technologies, Carlsbad, CA) and PureLink® Quick Plasmid Miniprep Kit (Life Technologies, Carlsbad, CA). Plasmids were quantified using the NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE) and were serially diluted from 106 to 100 copies for creation of a standard curve.

Duplex real-time PCR assay for simultaneous MG and ILT detection: Real-time was performed for all extractions on the Applied Biosystems® 7500 Fast Real-Time PCR System using a 25µL assay of 12.5µL 2X QuantiFast Multiplex Probe PCR Master Mix with ROX dye, 1.25µL mglp164 reverse primer (12.5µM), 1.25µL mglpU26 forward primer (12.5µM), 1.25µL mglp probe (1µM) (fluorescent label changed from FAM to Cy5; 3'BHQ®-2 (Integrated DNA Technologies, Coralville, IA) added), 1.25µL gC reverse primer (12.5µM), 1.25µL gC forward primer (12.5µM), 1.25µL gC probe (1µM)(FAM labeled), and 5µL template DNA. Primers and probes for MG and ILT real-time have previously been developed (1, 2). The thermal profile used for duplex ILT and MG amplification was as follows: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C, 15 sec; 60°C, 60 sec with optics ON. CT values less than 37 for MG and less than 35 for ILT were considered for analysis.

Duplex real-time PCR assay for simultaneous MS and ILT detection: Real-time was performed as

discussed above using a 25µL assay of 12.5µL 2X QuantiFast Multiplex Probe PCR Master Mix with ROX dye, 1.25µL MS ISR reverse primer (12.5µM), 1.25µL MS ISR forward primer (12.5µM), 1.25µL MS ISR probe (1µM) (fluorescent label changed from FAM to TAMRA; TAOTM and 3'IB®RQ (Integrated DNA Technologies, Coralville, IA) added), 1.25µL gC reverse primer (12.5µM), 1.25µL gC forward primer (12.5µM), 1.25µL gC probe (1µM)(FAM labeled), and 5µL template DNA. Primers and probes for MS real-time have also previously been developed (3). The thermal profile used for duplex ILT and MS amplification was identical to the profile used above for the MG/ILT duplex. Again, CT values less than 37 for MS and less than 35 for ILT were considered for analysis.

Sensitivity and specificity testing: For sensitivity testing, the serial 10-fold dilutions of plasmids (106 to 100 copies) for target genes of MG, MS, and ILT were run in triplicate on both duplex real-time PCR assays and were analyzed against separate routinely used assays for MS, MG, and ILT detection (1, 2, 3). The novel duplex real-time PCR assays were analyzed for specificity of the targets by running the assays against a number of avian mycoplasmas (pathogenic and non-pathogenic), ILT, and other relevant poultry respiratory pathogens, such as IBV and NDV.

RESULTS

Tracheal samples. Figure 1 (A&B) show log10MCN between singlet and duplex real-time PCRs. There were no discrepancies between positive and negative results of the duplex PCR and the established individual real-time PCRs for all tracheal samples. There were no significant differences ($P<0.05$) between singlet and duplex real-time PCRs for ILT, MG, or MS detection.

Sensitivity and specificity. Both duplex assays exclusively amplified their intended targets; there was no amplification of any other poultry pathogens using the assays. The detection limit for the MS/ILT duplex was 100 copies and the detection limit for the MG/ILT duplex was also 100 copies, indicating that the duplex assays were sensitive for the detection of their intended targets. Average CT values for each assay target at specific genome copy numbers, linear equations, and R2 values can be seen in Table 1.

DISCUSSION

In concluding this study, duplex real-time PCR assays have been validated for simultaneous detection of MG or MS with ILT in broilers showing no significant differences in detection of the pathogens compared to individual, previously established assays. The two duplex assays were both specific and sensitive for the detection of the intended targets. Use of this duplex real-time PCR will reduce the monetary and time-based costs for detection of these infections in a diagnostic setting. Future developments will include detection these three pathogens with avian influenza in a single panel for avian respiratory diseases.

REFERENCES

1. Callison SA, Riblet SM, Sun S, Ikuta N, Hilt D, Leiting V, Kleven SH, Suarez DL and García M. Development and Validation of a Real-Time Taqman® Polymerase Chain Reaction Assay for the Detection of Mycoplasma gallisepticum in Naturally Infected Birds. Avian Dis. 2006; 50:537-544.

2. Callison, S. A., Riblet, S. M., Oldoni, I., Sun, S., Zavala, G., Williams, S., ... & Garcia, M. (2007). Development and validation of a real-time Taqman® PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. Journal of virological methods, 139(1), 31-38.

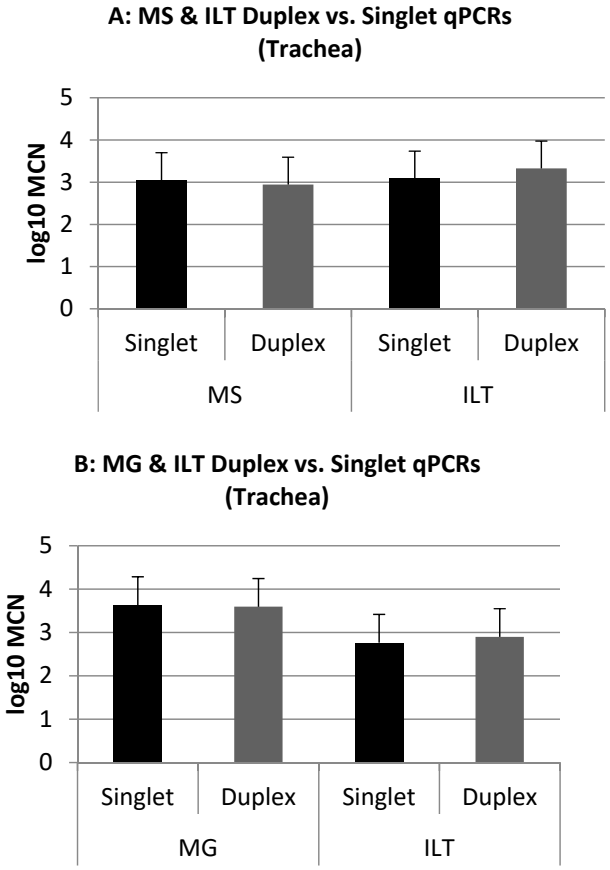
3. Raviv, Z., & Kleven, S. H. (2009). The development of diagnostic real-time TaqMan PCRs for the four pathogenic avian mycoplasmas. Avian diseases, 53(1), 103-107.

Table 1.

Target Gene Copy Number	Average C_T Values (±S.E.M) MS	ILT (MS Duplex)	MG	ILT (MG Duplex)
10^6	23.30±0.07	19.41±0.04	22.52±0.03	19.61±0.02
10^5	26.64±0.02	22.72±0.02	25.91±0.03	22.97±0.01
10^4	29.64±0.04	26.12±0.03	29.33±0.04	26.26±0.01
10^3	31.71+0.08	29.20±0.01	32.35±0.12	29.69±0.05
10^2	35.04±0.21	32.36±0.26	35.04±0.17	32.68±0.10
10^1	b	35.33±0.40[a]	b	37.56±2.19[a]
10^0	b	b	b	b
Linear equation	y=-0.348x+14.197	y=-0.317x+13.199	y=-0.313x+12.127	y=-0.285x+11.509
R^2 value	0.995	0.998	0.999	0.995

a=2/3 positive; b=none positive

Figure 1.



INCREASED INCIDENCE OF ENTEROCOCCUS ISOLATIONS FROM NON-VIABLE CHICKEN EMBRYOS IN WESTERN CANADIAN HATCHERIES AND EFFICACY OF BACTERIAL IDENTIFICATION BY MATRIX ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

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ABSTRACT

The objective of this study was to identify bacteria associated with chicken embryonic mortality and evaluate efficacy of MALDI -TOF MS in bacterial identification. Hatch debris were collected at varying stages of embryonic development during incubation and analysed from three commercial hatcheries across western Canada over a one year period. Bacterial isolation was performed from yolk samples and bacterial identification was performed by MALDI-TOF MS. 66.31 % of the yolk samples were positive for at least one type of bacterial growth and Enterococcus species (~40%) was predominant followed by Escherichia coli (~30%). Of the Enterococcus species identified, *E. faecalis* was predominant (82.12%), followed by *E. faecium.* The MALDI-TOF MS identified 84.24% of bacteria to the genus level and 72.61% to the species level. According to our observations, MALDI-TOF MS is a reliable bacterial identification tool in poultry diagnostics. Role of Enterococcus spp in pathogenesis of embryonic mortality needs further research.

INTRODUCTION

Yolk sac infections in chicken embryos and neonatal chickens cause significant economic losses to poultry producers. *E.coli* was the predominant bacterial species isolated so far in yolk sac infections (4, 6). Bacteria which get access to incubating hatching eggs may can cause embryonic mortality and yolk sac infections in neonatal broiler chickens (1). Enterococcus spp were isolated in high numbers from yolk sac infection cases in recent past in western Canada. (Chicken Farmers of Saskatchewan, April 2015). Even though pathogenesis associated with enterococcus infections in human are characterised, the involvement of enterococcus species in chicken embryonic death and yolk sac infections are not yet studied. Though MALDI-TOF MS has been successfully and broadly applied for the identification of human pathogens, identification of poultry bacterial pathogens are still based on conventional biochemical methods and rarely used this technique in poultry diagnostics. So our study aimed to isolate and identify different bacterial species from dead in shell embryos and to evaluate the effectiveness of MALDI -TOF MS technique in identifying poultry bacterial isolates and to access future applicability in poultry disease diagnosis.

MATERIALS AND METHODS

Hatch debris which are unhatched eggs at 21 days of incubation period were collected from three hatcheries in western Canada. Breakout analysis was performed and bacteriological swabs from yolk samples were aseptically collected from randomly selected hatch debris. These swabs were cultured on 5% sheep blood agar (Oxoid, Nepean, Ontario, Canada) and incubated at 37˚C for 24 to 48 hours aerobically and anaerobically. Isolated bacteria were identified using MALDI TOF MS. Formic acid extraction was performed for the isolates which did not give reliable identification by direct transfer method. As specified by the manufacturer, identification scores of ≥ 2 and between ≥ 1.7 and < 2 were required for a secure reliable identification to the species and genus level, respectively, while identification scores of ≤ 1.7 were considered unreliable. Randomly selected isolates with reliable identification in to species level were subjected to cpn60 and/ 16s rRNAgene sequencing to determine accuracy of MALDI-TOF MS identification.

RESULTS

Breakout analysis revealed that majority of embryonic mortality occurred during the late stage (34.41%) followed by early (19.24%) stage of incubation. Third most common was infertile

(11.24%). This pattern was consistent with all three hatcheries. 66.31 % of the yolk samples were positive for at least one type of bacterial growth. It was evident that out of bacteria positive eggs, 35.85% were single type bacterial colonies and the rest (30.46%) were multiple type bacterial colonies. This growth pattern did not greatly vary with the breakout category from which yolk samples were collected. A total of 920 isolates were recovered from the yolk samples and none of the anaerobes were observed. The predominant bacterial species isolated was Enterococcus species (35.9 %) from the total isolates followed by *E.coli* (22.45%).Third most common isolate was Staphylococcus spp (12.91%). This trend was consistent with all three hatcheries. *Enterococcus faecalis* (82.12%)) was the predominant isolate among all Enterococcus species isolated followed by *Enterococcus faecium* (9.09%). Other than above major isolates, *E. avium*, *E.casseliflavus*, and *E.gallinarum* were isolated in low but different percentages. MALDI TOF MS was able to identify 84.24% (775/920) of isolated bacteria into secure genus level and 72.61% (668/920) of isolated bacteria into secure species level. Out of total isolates, 15.43 % (142/920) isolates did not get any reliable identification either from direct transfer method or formic acid extraction method. There was a 100% similarity between MALDI-TOF MS IDs & either cpn60 &/ 16s rRNAgene sequencing IDs for tested isolates to determine the accuracy in MALDI-TOF MS.

DISCUSSION

It is well established that *E. coli* is associated with yolk sac infection leading to embryonic death; however, isolation of high numbers of Enterococcus species from chicken dead embryos, as shown in this study, suggests that this organism may be considered a significant and emerging pathogen in the poultry industry. In fact, for the last few years, Enterococcus species have been isolated from a significant number of yolk sac infections in Western Canada hence, more studies are needed to understand the role of this organism in embryonic mortality. Recent developments in bacteriology such as real-time PCR, universal gene target sequence analysis, or microarray analysis for identification of bacteria do not provide the complete solution in routine bacterial identifications, since the rapid identification of etiological agents in infectious diseases is pivotal to guide the antimicrobial therapy (7) MALDI -TOF MS is an alternative to conventional biochemical techniques and molecular based methods which is very cost effective and having a rapid turnaround time (2,3,5) Even though MALDI TOF MS is frequently used in medical diagnostic laboratories, application in poultry diagnostics is really low. Our data suggest that MALDI-TOF MS can be used as an alternative to conventional diagnostic approaches as a robust, accurate and reliable bacterial identification platform for identifying poultry pathogens.

REFERENCES

1. Berrang, M. E., Cox, N. A., Frank, J. F., & Buhr, R. J. Bacterial penetration of the eggshell and shell membranes of the chicken hatching egg: a review. *The Journal of Applied Poultry Research*, *8*(4), 499-504.1999.

2. Bizzini, A., K. Jaton, D. Romo, J. Bille, G. Prod'hom, and G. Greub. Matrix-assisted laser desorption ionization-time of flight mass spectrometry as an alternative to 16S rRNA gene sequencing for identification of difficult-to-identify bacterial strains. Journal of clinical microbiology 49:693-696. 2011.

3. Carbonnelle, E., C. Mesquita, E. Bille, N. Day, B. Dauphin, J.L. Beretti, A. Ferroni, L. Gutmann, and X. Nassif. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clinical biochemistry 44:104-109. 2011.

4. Cortés, C.R., G.T. Isaías, C.L. Cuello, J.M.V. Flores, R.C. Anderson, and C.E. Campos. Bacterial isolation rate from fertile eggs, hatching eggs, and neonatal broilers with yolk sac infection. Revista Latinoamericana de Microbiologia 46:12-16. 2004.

5. Croxatto, A., G. Prod'hom, and G. Greub. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS microbiology reviews 36:380-407. 2012.

6. Munang'andu, H.M., S.H. Kabilika, O. Chibomba, M. Munyeme, and G.M. Muuka. Bacteria isolations from broiler and layer chicks in zambia. Journal of pathogens 2012:520564. 2012.

7. van Veen, S.Q., E.C. Claas, and E.J. Kuijper.High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. Journal of clinical microbiology 48:900-907. 2010.

SPATIO-TEMPORAL RISK MAPPING OF WATERFOWL MOVEMENTS AND HABITAT IN THE CENTRAL VALLEY OF CALIFORNIA USING NEXT GENERATION RADAR AND LANDSAT IMAGING AS A MECHANISM FOR GUIDING AVIAN INFLUENZA SURVEILLANCE

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OVERVIEW

Recent surveillance efforts for avian influenza viruses (AIv) in commercial poultry have addressed spatio-temporal correlation with migratory waterfowl. However, an additional component to AIv disease dynamics still neglected is the variability of waterfowl habitat, including natural and man-made wetlands. We are employing a multimodal spatio-temporal approach to track waterfowl and their habitat, which utilizes a combination of next generation radar (NEXRAD) and remote sensing (Landsat) data. This utilization of radar to track waterfowl with the intent to quantify infectious disease risk is a novel use of NEXRAD technology. In this work, the locations of high density waterfowl habitat derived from Landsat were combined with NEXRAD data to generate risk maps for AI transmission to commercial and backyard poultry in the Central Valley of California. In order to better understand waterfowl distribution and variability of habitat based on differing environmental conditions we analyzed quarterly waterfowl distribution and land cover data for years of severe drought (2014-2015), above average wetness (2005-2006), and for an average wetness year (2003).

INTRODUCTION

Detection of H5N8 and H5N2 strains of Highly Pathogenic Avian Influenza (HPAI) in British Columbia, Washington, Oregon and California in December 2014 underscores the importance of active AIv surveillance to protect commercial and backyard poultry in California. The primary reservoir for AIv – migratory waterfowl – are thought to introduce the virus during overwintering periods to naïve populations that exist near roosting and feeding sites (1, 4, 5, 6). Landsat can identify waterfowl habitat by detecting the spatial distribution of wetlands and flooded croplands. NEXRAD facilitates detection of waterfowl directly, through analysis of their coordinated evening feeding flights evident during overwintering periods (3). Proximity of waterfowl to commercial and backyard poultry represent the highest risk for transmission to domestic species, and thus the highest risk for large-scale AIv epidemics; especially in the northern half of the state where AI is most commonly identified (2). Traditional surveillance for AIv in high risk wildlife and backyard poultry is primarily passive and relies on submissions from the public, which do not take into account migratory patterns, seasonality, or changes in waterfowl habitat over time. Wetland distribution, and thus waterfowl habitat, can change dramatically with fluctuation in yearly precipitation, highlighted by drought conditions over the past decade within California. Tracking this habitat dynamic lends insights into the spatiotemporal patterns of migratory waterfowl and facilitates a more directed, active approach to AIv surveillance.

MATERIALS AND METHODS

Wet, dry, and average year were defined by yearly precipitation from the California Department of Water Resources. NEXRAD data from three radars (KBBX, KDAX, KHNX) were combined with wetness indices derived from Landsat 5 & 7, and National Agriculture Statistics Service (NASS) Cropland data to determine probable waterfowl roosting locations. These sites were juxtaposed with locations of commercial and backyard poultry, and all data imported into ArcGIS 10.3 for processing. Differences in spatiotemporal association were quantified between wet, dry and average wetness years.

RESULTS AND DISCUSSION

In order to develop a comprehensive understanding of the dynamics of waterfowl distribution relative to domestic poultry, mapping of commercial farms, backyard poultry, and waterfowl habitat was integrated with literature reviews to understand the behavior, distribution and range of movement. Results show highly variable space-time clustering of waterfowl within wetlands, which exhibited precipitation dependent distribution. Consistently low waterfowl density was noted in the southern San Joaquin/Northern Tulare Basins, which correlated with low prevalence of waterfowl habitat in those areas. Focusing of surveillance efforts in regions with high waterfowl density and AIv-susceptible populations may lead to more efficient, earlier detection of AIv in California.

CONCLUSION

Spatial and temporal mapping of geographical areas where waterfowl interface with domestic poultry was performed in order to better target AI surveillance efforts in California. The first step in understanding the risk of AI to domestic poultry is to understand the relationship between the agent, host, and the environment. Consequently, epidemiological tools including GIS and spatial statistics are essential toward understanding risk. In this project, the spatiotemporal intersection of commercial and backyard poultry operations with migratory wild waterfowl flyways, natural wetlands, and flooded agricultural fields in in the Central Valley of California was identified to assess risk for AIv transmission and how that risk may change over time with changing environmental conditions. Future directions include the development of machine learning models to predict waterfowl distribution beyond NEXRAD range, and real-time processing of NEXRAD and remote sensing data.

ACKNOWLEDGEMENTS

Central Valley Joint Venture and Ducks Unlimited for providing shapefiles of wetlands and crop locations within California. This work has been funded by a UC-ANR grant.

REFERENCES

1. Bahl, J., S. Krauss, D. Kühnert, M. Fourment, G. Raven, S. P. Pryor, L. J. Niles, A. Danner, D. Walker, I. H. Mendenhall, Y. C. F. Su, V. G. Dugan, R. A. Halpin, T. B. Stockwell, R. J. Webby, D. E. Wentworth, A. J. Drummond, G. J. D. Smith, and R. G. Webster. Influenza A Virus Migration and Persistence in North American Wild Birds. PLoS Pathog 9:e1003570. 2013.

2. Bevins, S. N., R. J. Dusek, C. L. White, T. Gidlewski, B. Bodenstein, K. G. Mansfield, P. DeBruyn, D. Kraege, E. Rowan, C. Gillin, B. Thomas, S. Chandler, J. Baroch, B. Schmit, M. J. Grady, R. S. Miller, M. L. Drew, S. Stopak, B. Zscheile, J. Bennett, J. Sengl, C. Brady, H. S. Ip, E. Spackman, M. L. Killian, M. K. Torchetti, J. M. Sleeman, and T. J. Deliberto. Widespread detection of highly pathogenic H5 influenza viruses in wild birds from the Pacific Flyway of the United States. Sci Rep 6:28980. 2016.

3. Buler, J. J., L. A. Randall, J. P. Fleskes, W. C. Barrow, Jr., T. Bogart, and D. Kluver. Mapping wintering waterfowl distributions using weather surveillance radar. PloS one 7:e41571. 2012.

4. Munster, V. J., and R. A. M. Fouchier. Avian influenza virus: Of virus and bird ecology. Vaccine 27:6340-6344. 2009.

5. Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. Osterhaus, and R. A. Fouchier. Global patterns of influenza a virus in wild birds. Science (New York, N.Y.) 312:384-388. 2006.

6. Peterson, A. T., B. W. Benz, and M. Papes. Highly pathogenic H5N1 avian influenza: entry pathways into North America via bird migration. PloS one 2:e261. 2007.

THE GENOMIC CONSTELLATION OF THE S1, S2 AND THE S3 GENE SEGMENTS FROM RECENT AVIAN REOVIRUS ISOLATES ASSOCIATED WITH VIRAL ARTHRITIS AND RUNTING-STUNTING CASES IN GEORGIA

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SUMMARY

Nucleotide sequences of the S1, S2 and the S3 class genome segments of 21 field isolates of ARV from broiler chicks over a period of four years were examined to define phylogenetic profiles, and their relationship with the isolates from the previous outbreaks. Three distinct lineages of the S1 gene were observed. Majority of the isolates showed high percentage of nucleotide identity with the recent isolates reported elsewhere in Georgia. The remaining isolates were clustered into two different groups, comprised of variants from previously reported outbreaks that shared 86-90% amino acid identity. All the isolates showed less than 50% amino acid identity with ARV vaccine strains like S1133. For the S2 and the S3 genes, most isolates shared 84% to 98% amino acid identity with each other as well as with vaccines strains, and at least, with two recent field isolates associated with runting-stunting syndrome and viral arthritis. Results of this study indicate that the recent isolates of ARV are unique to viral arthritis cases and are genetically different from the traditional ARV vaccines strains.

Avian reoviruses (ARVs) are widespread in nature and are associated with a wide range of diseases affecting avian species, including chickens. In young broilers they have been associated with various disease conditions such as viral arthritis, "runting-stunting" syndrome (RSS), malabsorption, immunosuppression and respiratory infections (5). Significant economic losses are due to increased mortality, diminished weight gain, general lack of performance, an uneven growth rate, and reduced marketability of the affected chicks. ARVs belong to the *Orthroreovirus* genus in *Reoviridae* family (2). ARVs contain 10 double-stranded RNA genome segments, including 3 L (large), 3 M (medium), and 4 S (small) size classes based on the segments' electrophoretic mobility (10). The outer capsid sigma C protein encoded by the S1 genome segment is the cell attachment protein and a major antigenic determinant for ARVs. This S1 genome segment of the existing strains as well as emerging strains is well characterized, and has been shown to exhibit the highest level of sequence variation when compared with other genes (11). The S2 gene segment encodes double-stranded RNA binding protein, sigma A, that may be involved in resistance to interferon (2). The S3 segment encodes the outer capsid protein, sigma B, which carries group-specific neutralizing epitopes (12).

Georgia broiler industry experienced recurrent outbreaks of RSS and viral arthritis since 2006. In this paper, we report S1, S2 and the S3 gene segment characterization of field isolates from broiler chicks from 2012 to 2015 outbreaks for better understanding the evolutionary lineages of newly emerging ARV field variants.

MATERIAL AND METHODS

Virus isolation and identification. Tendons, liver and intestines were homogenized in phosphate buffered saline supplemented with antibiotics. Crude virus suspension was clarified by centrifugation and filtered through a 0.2 um membrane filter, and used for inoculation of primary chicken embryo kidney (CEK) monolayers. The first two serial passages produced isolated foci of detached cells within 72 hours post inoculation. After the third passage, there was marked rounding and detachment of cells.

RNA isolation and RT-PCR. Viral RNA was extracted from the clarified supernatants of CEK-infected monolayers using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The amplification of the sigma C was performed using the One-step RT-PCR kit (Qiagen, Valencia, CA) and previously described primers (6). The S2 and S3 gene segments were amplified using primers described by Banyai et al (1). The RT-PCR products of the virus were directly sequenced using the same set of primers. Sequencing reaction was performed using 30-ng template per reaction with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) at the Georgia Genomics Facility, UGA, Athens, GA.

Sequence analysis. Sequence information was compiled with the SeqMan program (Lasergene v9.0, DNASTAR, Madison, WI). The alignment of sequence data and the construction phylogenetic tree

was performed by MegAlign program (v9.0, DNASTAR) using the Clustal W multiple sequence alignment algorithm (7). The reovirus reference strains from the US that showed high similarity in basic local alignment search tool (BLAST) were selected for analysis. In addition, commonly used US vaccine strain S1133 was also included in the analysis. The GenBank accession numbers of various isolates used for analysis are listed in Figure 1 and Table 1.

RESULTS

ARV isolation and identification. Most field isolates came from tendons of affected chickens of 10 to 26 days of age. Cytopathic effect in CEK was characterized by the formation syncytia within 48 to 72 hours after inoculation. Isolates were successfully amplified using S1, S2 and S3-specific primers to obtain the PCR product of approximately 1kb.

Phylogenetic analysis of the ARV isolates. For molecular analysis of the S1 segment, a total of 13 ARV field isolates from 2011-2015 cases of viral arthritis and another 11 ARV reference sequences retrieved from GenBank were included. Genotype comparison also included reovirus vaccine strains S1133, 1733 and 138. Similarity of the genotype of field isolates was reported out as the highest percentage amino acid (aa) similarity to another sequence. All the isolates were grouped into three distinct phylogenetic groups (Figure 1). Of the 13 field isolates, six were (bold face letters) in the same cluster (Group 3) as the variants reported elsewhere in Georgia during 2012 (9). All the isolates within this group showed high bootstrap nodal values and short branching distances indicating they are of similar origin. The sigma C aa sequences of ARVs from this group were 98% to 100% (Table 1) similar to each other as well as with the reference isolates, KJ879709, KJ803982, and KJ 879699. In Group 2, three isolates were grouped with the reference isolates that were reported from Georgia during 2006 and 2012 wide-spread occurrences of runting-stunting cases (3). These isolates although, shared aa identity of 86 to 92% with DQ872797, KJ879686 and JX983600 reference strains (Table 1), were genetically distinct from each other. In Group 1, the remaining four isolates (KY373264, KY373268, KY373272, and KY373273), were grouped together with KR856977 JX983602, JX983599, and KJ8796282. These reference isolates were derived from RSS as well as VA cases reported previously. Although the branched distances were slightly higher than those from Group 1, the nodal values were high indicating a similar origin. Amino acid identities of all the four isolates within this group ranged between 89-92% (Table 1). Variants from all the three groups were <60% similar to each other (data not shown). Interestingly, all the isolates from the present study shared< 50% aa identity with classic ARV vaccine strains (S1133, data not shown).

For the S2 gene, deduced aa sequences of eight isolates shared wide range of identity (84 -99%, Table 1) with each other. These isolates also showed similar levels of identity with S1133 (KF741763), 138 (AF059717), and with those from RSS (FR694198) and VA cases (KM877331). Similarly for the S3 gene, all the isolates shared 96-99% identity with each other, with S1133 (KF741764), 138 (AF059721) as well as the isolates from RSS (FR694199) and VA outbreaks (KM877333), (Table 1).

DISCUSSION

In this study, we compared the nucleotide sequences of the S1, S2 and S3 gene segments of ARV isolated from Georgia for the past four years. Since the initial isolation reovirus virus from RSS cases (GenBank accession no. DQ872797) in 2006, there were recurrent isolations of ARV from tendons of chicks affected with lameness. Most isolations came from broiler chicks of 10- 28 days of age. Control of reovirus-induced arthritis is achieved by vaccination of broiler breeders with a combination of live and/or inactivated vaccines, thereby providing progeny with specific maternal antibodies to protect from early challenge with field variants. In broilers, live attenuated vaccines are also available for day old chicks. During the intense reovirus activity in 2012, only few commercial vaccines strains (S1133, 1733 or 2177) were available. This raised the possibility of large-scale vaccine failures due to emergence of variants. Earlier studies in 2013 have shown that these variants were indeed genetically and antigenically distinct from current reovirus vaccine strains (3, 9). In the present study, we extended these observations to more recent cases of viral arthritis to confirm these findings.

Until recently, sequence data from few classic ARV strains (S1133, 138, and AVS-B) were available from public databases. The most variable sigma C protein, displays the highest level of sequence divergence (8). Therefore, the sigma C gene could be used as genetic marker for classification of ARV. This outer minor capsid protein is responsible for cell attachment and induction of type-specific neutralizing antibodies (12). In the present study, three distinct phylogenetic groups were identified. Seven out 13 isolates analyzed, belonged to Group 1 and 2 described earlier (3). Reference isolates clustered within these two groups were from RSS cases of 2006 (DQ872797, FR694197), and 2012 (JX983602, JX983599, and JX983600). Interestingly, 2012-2013 isolates from Georgia and Pennsylvania (KJ8796282, KJ879686, and KR856977) also clustered in these two groups,

indicating that similar isolates of ARV might be circulation in the three states. Six out of 13 sequences of the sigma C gene that clustered in Group 3 showed the highest genetic identity only with the recent isolates from several other states including those from Georgia. Lack of similarity of these variants with vaccine strains, is consistent with the chick inoculation studies on Group 1 Georgia variants (2012 VA variants) described elsewhere (9). *In vitro* studies from this report indicated that current commercial vaccines do not provide protection against new variants. However Group 1 and Group 2 isolates from the present study, differ from the Group 2 variants described by this author. Our isolates from these two groups shared 86-92% similarity with each other in contrast to >99% , reported by Sellers (9). Apparent reason for this is not known.

The new geneotype cluster represents emerging ARV variants in the US. These findings are consistent with the earlier suggestion that ressortment between reoviruses of RSS and arthritis could have played a role in rapid emergence of Georgia variants, probably due to high population of susceptible broiler chicks (4, 11). Thus the marked divergence amongst field variants reported from several states has affected the efficacy of ARV vaccines routinely used by the poultry industry. In contrast to the S1 gene, the S3 gene, which encodes outer capsid protein, however shared 94-99% similarity with each other as well as with the reference strains.

In conclusion, new ARV variants exhibit a new genotype (s) described so far. We have demonstrated that with every recurrent outbreak due to ARV, new groups of variants emerge, which show little or no similarity with the previously characterized isolates. Diversity of the S1 gene into one or more phylogenetic groups and sequence pairwise comparison results provided evidence that accumulation of point mutations throughout this gene may have contributed toward the emergence of variants described here. These studies will help to choose appropriate candidates for developing effective autogenous killed-virus vaccines.

REFERENCES

1. Banyai, K., Dandar, E., Dorsey, K.M., Mato, T., and Palya, V. The genomic constellation of a novel avian orthoreovirus associated with runting-stunting syndrome in broilers. Virus Genes, 42:82-89, 2011.

2. Benavente, J., and Martinez-Costas, J. Avian reovirus: Structure and biology. Virus Research, 123:105-119, 2007.

3. Davis, J., Kulkarni, A., and Fletcher, O. Reovirus infections in young broiler chicks. Avian Dis. 57:321-325, 2013.

4. Day, M.J. The diversity of the orthoreoviruses: Molecular taxonomy and phylogenetic divides. Infection, Genetics, and Evolution. 9:390-400, 2007.

5. Jones, R. C. Viral Arthritis. In: Diseases of Poultry, 12th ed, Y. M. Saif, A. M. Fadly, J. R. Glission, I. R. McDougald, L. K., Nolan, and D.E. Swayne, eds. Blackwell Publishing, Oxford, United Kingdom, pp 310-322, 2008.

6. Kant, A., F. Balk, L. Born, D. van Roozelaar, J. Heijmans, A. , Gielkens, and A. Huurane. Classification of Dutch and German avian reoviruses by sequencing the □C protein. Vet. Res. 34: 203-212, 2003.

7. Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T. J. Gibson, and D. G. Higgins. Clustal W and Clustal IX version 2. Bioinformatics: 23 (21): 2947-2948, 2007.

8. Liu, H.J., Lee, L.H., Hsu, H. W., Kuo, L. C., and Liao, M. H. Molecular evolution of avian reovirus:evidence for genetic diversity and ressortment of the S-class genome segments and multiple cocirculating lingeages. Virology 314:336-349, 2003.

9. Sellers, H. S. Update on variant reoviruses isolated from clinical cases of viral arthritis/tenosynovitis in broilers. The Poultry Informed Professional (published by the Department of Population Health, University of Georgia, Athens, GA), 127 (Jan/Feb): 1-4, 2013.

10. Spandidos, D. A., and Graham, A. Physical and chemical characterization of avian reovirus. J. Virol. 19:968-976, 1976.

11. Tang, Y and Lu H. Genomic characterization of a broiler reovirusfield strain detected in Pennsylvania. Infection, Genetics, and Evolution. 31:177-182, 2007.

12. Wickramasinghe, R., Meanger, J., Enriquez, C.E., and Wilcox, G.E. Avian reovirus proteins associated with neutralization of virus infectivity. Virology, 194: 688-696, 1993.

Figure 1. The deduced amino acid sequences of thirteen ARV isolates from broiler chicks were aligned with Clustal W algorithm and the phylogenetic tree was constructed using the neighbor joining method (7). The analysis was conducted using DNASTAR v9.0. GenBank accession numbers of the isolates used in this study are shown in bold-face fonts.

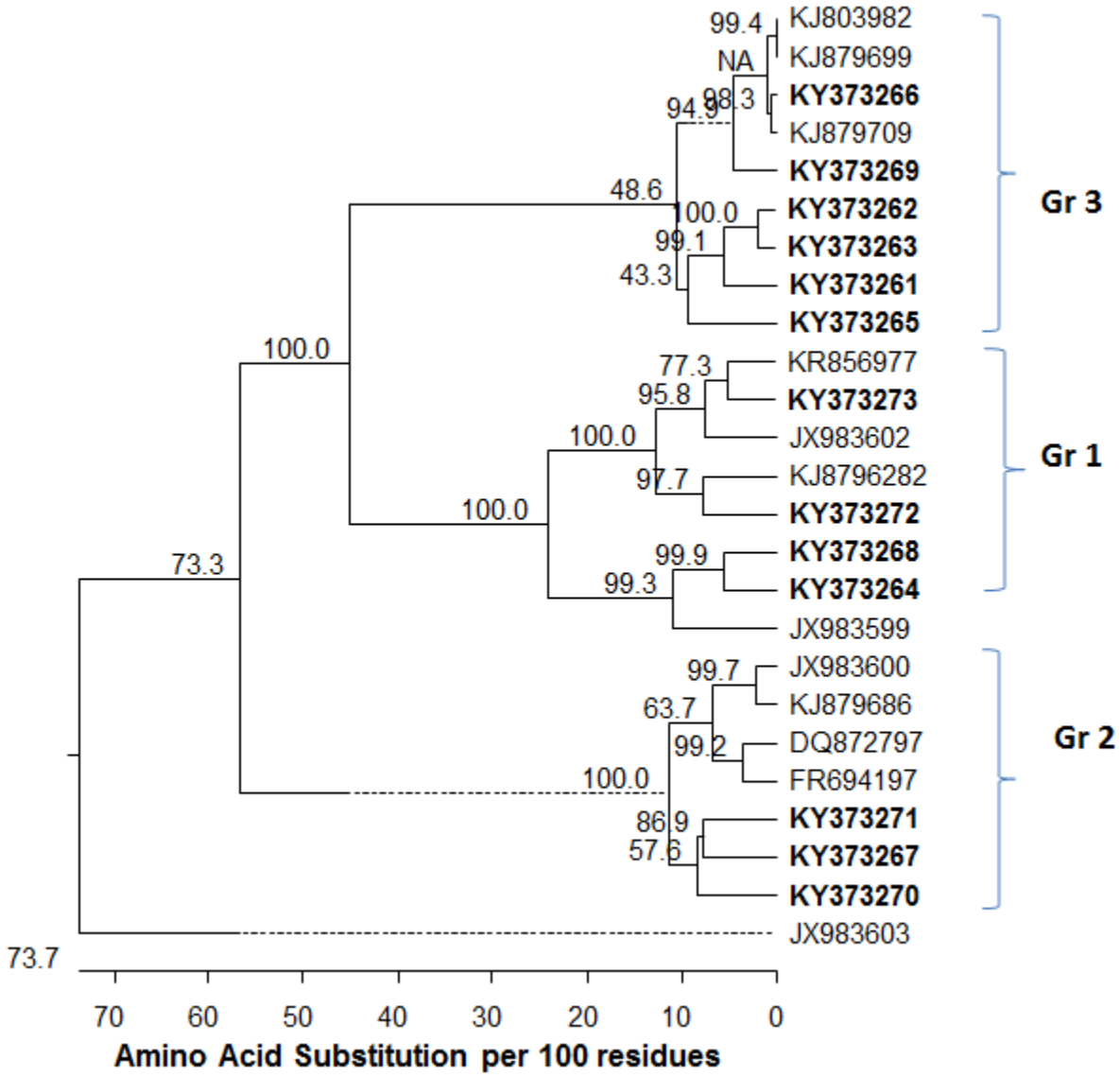

Table 1. Summary of amino acid identity of the S1, S2 and the S3 gene segments of ARV isolates analyzed in this study.

Gene segment	GenBank accession no. from this study	Phylogenetic group	GenBank accession number of reference isolates showing % amino acid identity
S1	KY373261 KY373262 KY373263 KY373265 KY373266 KY373269	3	KJ879709, KJ803982, KJ879699 98-100%
S1	KY373271 KY373270 KY373267	2	FR694197, DQ872797, JX9836600,
S1	KY373264 KY373268 KY373273 KY373272	1	KJ879686 86-92% JX983599, JX983602, KJ879682, KR856977 89-92%
S2	KY441642 KY441643 KY441644 KY441645 KY441646 KY441647 KY441648 KY441649	No groups	AF059717 (138), FR694198 (AVS-B), KF741763 (S1133) KM877331 84-99%
S3	KY441650 KY441651 KY441652 KY441653 KY441654 KY441655 KY441656 KY441657	No groups	AF059721 (138), FR694199 (AVS-B), KF741764 (S1133), KM877333 96-99%

NEXT GENERATION SEQUENCING APPLICATIONS IN POULTRY VIRAL AND BACTERIAL DISEASES

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Next Generation Sequencing (NGS) developed in the mid-2000s born out of the sequencing of the human genome project is now making significant impacts in Human and Veterinary Health. NGS has shortened the DNA sequencing of a single human genome from 13 years involving thousands of scientists around the world with greater than three billion dollars, to a few days within a week with just approximately $1000 sequencing reagents cost. The grand challenge of even lower DNA sequencing cost of $100 genome has been met has just been announced and will definitely be a game changer for medical and veterinary to health care sectors promising delivery of a better diagnosis and therapy platform technology.

NGS has already making great strike in human medical health through sequencing and one of the fields has been impacted the most is infectious viral and bacterial diseases; from genotyping and tracking viral and bacterial infections with whole/full viral and bacterial genome sequencing, and applying such knowledge to generating better genotyped matched vaccine development. Such promise is already being realized in human infectious (viral and bacterial) diseases and starting to make significant reroute in veterinary infectious diseases. I will discuss the promises of NGS and the challenges animal/poultry scientists, pathologists and veterinarians face in attempting to bring genomic sequence to the real-time needs of poultry diagnostic laboratories.

COMPREHENSIVE SERVICE AND DIAGNOSTICS PROGRAM AND ITS IMPLEMENTATION TO ELUCIDATE THE CAUSE OF INCREASED MORTALITY AND AIRSAC CONDEMNATIONS IN A BROILER PRODUCTION COMPLEX: A CASE REPORT

J. Linares, H. Roh, R. Sanchez, J. ElAttrache, and M. Putnam

SUMMARY

In this case report, a broiler production complex experienced increased mortality and airsac condemnations. Their respiratory vaccination program consisted of a new Infectious Bronchitis (IB) GA08 vaccine, plus Ark and B1/B1 Newcastle Disease (ND) administered at day old in the hatchery and field boost at 14 days. The new IB GA08 vaccine has label claims against GA08, GA13 and DMV1639. This broiler complex had a historical high prevalence of DMV1639 challenge.

A comprehensive service and diagnostics program was developed to evaluate and optimize the use of the new IB GA08 vaccine. The service and diagnostics program included the evaluation of hatchery vaccination and field boosting procedures, evaluation of vaccine reactions, vaccine detection via qRT-PCR and sick bird monitoring (10 flocks; clinical observations, acute and convalescent serology, necropsy, bacteriology, virology). Vaccine detection via qRT-PCR is a method to evaluate IB vaccine delivery to the birds. Tracheas from 15 chicks per farm were tested individually and the results are expressed as the percentage of positive tracheas in each group of 15 samples. A result ≥70% positive indicates good vaccine delivery. The service and diagnostics program was implemented to help elucidate the cause(s) of the problem.

RESULTS

Vaccine reactions. Post-hatchery and post-field boost vaccine reactions were unremarkable to mild.

Vaccine detection. Collected and tested individual tracheas from 15 chicks from each of 10 farms. See results on Table 1.

Clinical observations. During initial clinical observations it was established that there was respiratory noise in flocks ≥27 days-old and increased mortality in flocks ≥35 days-old. Based on these observations, ten flocks with ages 27-32 days-old, were examined for further diagnostic evaluation.

Necropsy. Airsac suds, pulmonary congestion and edema, and whitish moist fibrinous polyserositis (acute lesions) were observed in some of the birds with respiratory noise. Most dead birds had yellowish dry fibrinous polyserositis (chronic lesions).

Serology. Acute serum samples were collected at the onset of clinical signs and convalescent serum samples were collected just prior to processing. IBV ELISA results were consistent with uniform seroconversion to vaccination. NDV ELISA results were consistent with variable seroconversion suggestive of inconsistent immunization. ORT ELISA results were consistent with uniform seroconversion, hence challenge.

Bacteriology. ORT was isolated from acute lesions and *E. coli* was isolated from chronic lesions.

Virology. ND vaccine was isolated from flocks with respiratory signs post field boost. IBV isolates were characterized as GA 08 and Ark vaccine. IBV DMV1639 was isolated from a single flock.

CONCLUSION

Vaccine detection, serology and virology results were consistent with satisfactory IB immunization. The prevalence of IB DMV1639 was reduced.

Post-field boost ND vaccine-associated reactions, *Ornithobacterium rhinotracheale* and *E. coli* were identified as the main causes of increased mortality and airsac condemnations.

Table 1. Vaccine detection results.

Farm	Age (days)	qRT-PCR on trachea samples	
		% Positive IBV (5UTR)	% Positive GA08 Vaccine
A	4	100%	80%
B	5	100%	100%
C	5	100%	100%
D	7	100%	80%
E	7	100%	100%
F	11	100%	100%
G	11	100%	100%
H	12	100%	90%
I	13	100%	90%
J	13	100%	90%

A CASE OF DUCK VIRAL ENTERITIS IN BACKYARD MUSCOVY DUCKS

G. Lossie, P. Wakenell, and T. Lin

INTRODUCTION

Duck viral enteritis (DVE), also known as duck plague, is an acute, contagious, viral disease of ducks, geese, and swans (3). There is a wide range of susceptibility among duck species with blue-winged teals (*Anas discors*) being the most susceptible and the Northern Pintail duck (*Anas acuta*) being the most resistant (3). A single epornitic event occurred in wild birds at Lake Andes, South Dakota, in 1973 causing the death of 43,000 ducks and geese out of 100,000 birds residing at the lake (3). In domestic breeds, Muscovy ducks are extremely sensitive when compared to other breeds, and in multiple cases (including this one), are the only breed affected on a given premise (1, 2, 3). Clinical signs associated with DVE vary depending on the age of exposure. In ducklings, signs of diarrhea, a blood stained vent, dehydration, and a cyanotic bill appear three to seven days after exposure (4). In breeding flocks, males may have prolapse of the penis whereas females have a marked drop in egg production at the time of peak mortality (3). Mature birds are often found dead in good body condition (3). Clinical signs in mature birds include: photophobia, inappetence, extreme thirst, droopiness, ataxia, ruffled feathers, nasal discharge, soiled vents, and watery diarrhea (1).

CASE HISTORY

Several Muscovy ducks in a mixed breed and species flock died with clinical signs of cyanosis and bloody feces. Similar mortality in Muscovy ducks was reported on the premises the previous fall. There were no recent additions of birds to the flock or changes in animal husbandry. Domestic Mallard and Pekin ducks on the same premise were healthy with no signs of disease. A pond, rarely frequented by wild waterfowl, was maintained on the premise. Two, three month old Muscovy ducks were submitted to the diagnostic lab for necropsy.

Gross necropsy. There was mild pasting of the vents with green to white feces and urates. The vent exuded a moderate amount of thin, bloody, diarrheic fluid upon abdominal palpation. Mild to moderate amounts of blood stained mucous were present in the oral cavity of one duck. The livers were accentuated by numerous, multifocal, off white, foci measuring approximately one mm in diameter with scattered petechial to ecchymotic hemorrhages. Both birds had mildly swollen, 1.5 times larger than normal, dark red to black, spleens. The epicardial adipose tissue of both hearts had multifocal areas of mild petechial hemorrhage. The esophageal mucosa was covered in an off white to yellow, thin (less than one mm), necrotic membrane with diffuse, mucosal, petechial hemorrhage in one bird **(**Figure 1**)**. The proventricular mucosal surface was accentuated by strands of hemorrhage in one bird. The koilin layer of the ventriculus in a single bird was friable, dark red, and necrotic with clear lines of demarcation separating affected and non-affected portions. Coelomic fat stores in both birds had multifocal areas of petechial hemorrhage **(**Figure 2**)**. The pancreases were diffusely off white to pink with multifocal, areas of petechial hemorrhage **(**Figure 2**)**. The intestines, from duodenum to colon, in a single bird were diffusely dark red to black, thin walled and contained thin, bloody, digesta. The remaining duck had dark brown to green, thin walled, intestines with occasional intraluminal strands of hemorrhage. The cloacal mucosa in one bird was covered in a diphtheritic necrotic membrane.

Histopathology. The hepatic parenchyma contained multifocal to coalescing, random areas of necrosis comprising up to 80 percent of the total tissue. The spleen contained areas of multifocal fibrinoid necrosis with lymphoid depletion of the white pulp, which contained numerous hemosiderin laden macrophages. Large sections of the crop and esophageal epithelium were effaced by a membrane of necrotic debris that consisted of necrotic and degenerating heterophils and epithelial cells. Remaining intact epithelium was infiltrated by heterophils and variably ulcerated or lifted off from the underlying mucosa. Low numbers of intact epithelial cells contained eosinophilic, 2.5 mm in diameter, intracytoplasmic, and more rarely intranuclear inclusion bodies that were rarely surrounded by a thin, clear space or "halo." The cloacal mucosa mirrored the changes observed in the esophagus. The intestines (small and large) showed moderate to marked necrosis of the mucosal epithelium with variable effacement of normal parenchyma. The epicardial adipose tissue contained multifocal areas of mild hemorrhage.

Further diagnostics. Based on the gross and histopathologic findings a presumptive diagnosis of duck viral enteritis was reached. Sections of liver were sent to the National Veterinary Services Laboratory

for polymerase chain reaction (PCR) testing. PCR testing confirmed the diagnosis of duck viral enteritis.

DISCUSSION

This case is particularly relevant to poultry practitioners as recognition of this condition should prompt notification of the state wildlife department, department of natural resources, or board of animal health due to the serious implications of the disease to domestic and wild waterfowl. Prevention is based on keeping wild waterfowl off of ponds used by domestic birds, and limiting exposure to potentially contaminated water (3). Vaccination has been used as an effective preventative measure but can also be used in the face of an outbreak (3). There is no treatment available for DVE. Once a site is infected, control can be achieved via depopulation, removal of birds from the infected premise, cleaning and disinfection of the premise, and vaccination of susceptible ducklings (3).

REFERENCES

1. Davison S, Converse KA, Hamir AN, et al. Duck Viral Enteritis in Domestic Muscovy Ducks in Pennsylvania. Avian Dis 37: 1142-1146. 1993.

2. Campagnolo ER, Banerjee M, Panigrahy B, et al. An Outbreak of Duck Viral Enteritis (Duck Plague) in Domestic Muscovy Ducks (*cairina moschata domesticus*) in Illinois. Avian Dis 45: 522-528. 2001.

3. Metwally SA. Duck Virus Enteritis (Duck Plague). In: Diseases of Poultry 13th ed. D Swayne ed. John Wiley & Sons Inc, Ames. pp 431-440. 2013

4. Ojkic D, Brash ML, Jackwood MW, et al. Duck Virus Enteritis. In: Avian Disease Manual. 7th ed. Boulianne M, et al eds. AAAP, Inc, Jacksonville. pp 42-43. 2013

(Full case write up will be submitted to JAVMA "Pathology in Practice.")

Figure 1. Photograph of the mucosal surface of the esophagus. Not the raised, linear, yellow, necrotic, plaques covering the mucosal surface. Near the center of the image is a prominent band of petechial hemorrhage.

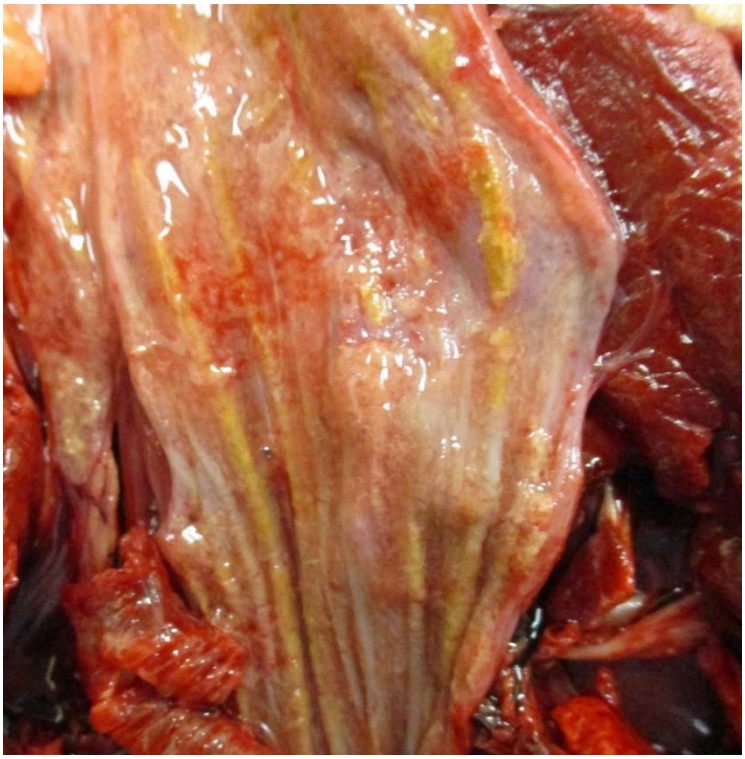

Figure 2. Photograph of the intestines, pancreas, and coelomic fat. There is prominent petechial hemorrhage throughout the coelomic fat. The pancreas is pale and off white with prominent pink mottling.

USE OF VECTORMUNE ND AS PART OF A COMBINED VACCINATION PROGRAM IN LAYERS FOR THE CONTROL OF VELOGENIC NEWCASTLE DISEASE IN A PREVIOUSLY REPORTED REGION IN MEXICO

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Ceva Animal Health -[A] Mexico; [B] United States

ABSTRACT

The objective of this study was to assess the protection against velogenic Newcastle disease (ND) using a combined Newcastle vaccination program in commercial layers in a previously reported region in Mexico. This combined vaccination program included a rHVT-F vector vaccine in conjunction to the adjusted conventional live and inactivated vaccines having the advantage of significatively reducing the number of vaccinations (From 18 live vaccinations to 14 and from eight oil emulsion vaccines to three in the 84 weeks of the layer's life). Three virulent ND challenges using a velogenic Newcastle Mexican strain (Chimalhuacan - Genotype V) was performed at 25 weeks of age and observed for 20 days. The parameters evaluated included clinical signs, mortality, RT-PCR in tracheal and cloacal swabs and serological response by Hemagglutination inhibition test. Protection of 100% was consistently observed in the vaccinated birds in all three virulent challenges. Egg weight measurements increased during the 20 day observation period.

INTRODUCTION

Velogenic Newcastle has been previously reported in some regions in Mexico affecting broilers, layers and breeders. Different ND vaccination regimes are applied for the control of this disease besides strict biosecurity. The objective of this study was to assess the synergistic protection given by a vector vaccine (rHVT-F) in addition to the adjusted conventional live plus inactivated ND vaccines reducing significatively the number of vaccinations. Furthermore, the protection against the virulent challenge to layer birds was measured by the increase in egg weight during the challenge period showing the protective synergistic effect of the combined vaccination program.

MATERIALS AND METHODS

Birds. A total of 60 Commercial Hy-Line Brown layers raised under commercial conditions from multiple age farms were randomly taken at 25 weeks of age and delivered to an isolation facility for virulent NDV challenge. Additionally, a total of 15 SPF birds were also used as controls for the challenge.

Vaccination program. A conventional live plus inactivated ND vaccination program was redesigned adding a vector rHVT-F recombinant vaccine (Vectormune ND) administered via sub-cutaneous route at day of age and combined with an adjusted and reduced number of conventional ND live and inactivated vaccines.

Challenge. Virulent ND challenge at 25 weeks of age using the Chimalhuacan strain (Genotype V) at a minimal dose of 10^5 $EID_{50\%}$ in 0.2 mL via eye drop and birds were observed for 20 days for clinical signs, mortality and egg mass.

Serological test. Hemagglutination inhibition test done at the start and end of the ND virulent challenge period.

Virus shedding. Measured by RT-PCR in the tracheal and cloacal swabs at three days post-challenge.

Egg weight measurement. Eggs were collected and their weight mass was measured at 5, 10, 15 and 20 days post-challenge.

CONCLUSION

The use of a vector rHVT-F recombinant vaccine as part of a combined ND conventional live and inactivated vaccines provided consistently 100% protection after virulent challenge to vaccinated commercial layers raised under commercial conditions in an endemic velogenic ND region in Mexico. The very virulent NDV challenge with a Chimalhuacan strain was given in isolated facilities at 25 weeks of age and observed for a 20 day-period. The egg mass measured at 5, 10, 15 and 20 days during the observational challenge period increased showing the synergistic protective effect of the combined ND vaccination program.

REFERENCE

1. Palya V. et al. Advancement in Vaccination Against Newcastle Disease: Recombinant HVT NDV

Provides High Clinical Protection and Reduces Challenge Virus Shedding with the Absence of Vaccine Reactions. 2012. Avian Diseases 56: 282-287.

Figure 1. Protective results after Virulent NDV challenges at 25 weeks of age were observed after 3 challenges.

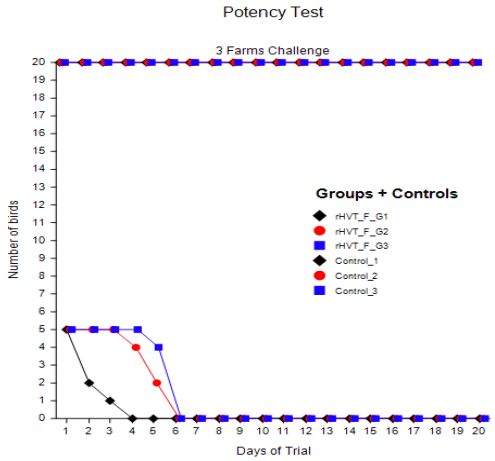


Figure 2. Egg weight measurements after 3 NDV virulent challenges assessed at different days post-challenge.

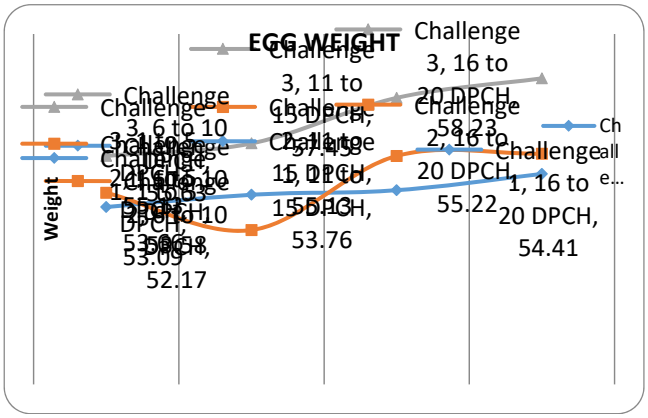


Figure 3. Results of RT-PCR in tracheal and cloacal swabs in each NDV virulent challenge at 3 days post-challenge showing no NDV shedding detected in the tracheal or cloacal swabs of the vaccinated layers compared to controls.

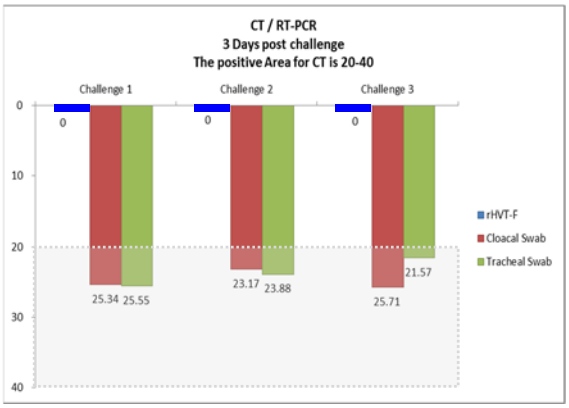
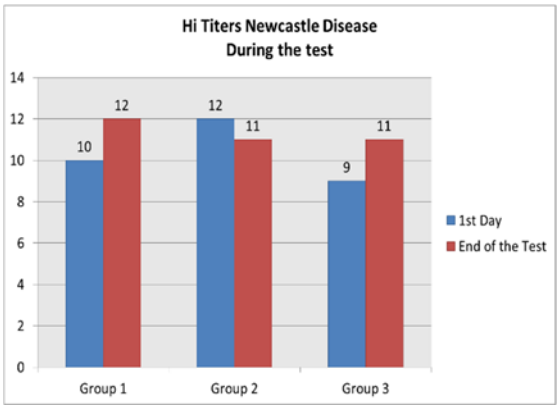


Figure 4. Newcastle HI titers obtained the day of the challenge and at the end of the challenge after 20 day period.



IMPLEMENTATION OF SEROLOGICAL BASE LINES BY REGION AND BY DISEASE FOR HEALTH MONITORING IN BROILER BREEDERS IN MEXICO

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ABSTRACT

The objective of this study was to generate reference values for the evaluation of vaccination programs in broiler breeders as well as for monitoring the health status and immune response to vaccination against four diseases during rearing and production phases. These serological base lines were established from samples taken from three different poultry producing regions of Mexico, starting from the day of age until 50 weeks of age. A similar vaccination program was administered to broiler breeders in these three geographic regions analyzed. Serological monitoring allowed the evaluation of vaccination quality at hatchery level as well as in the field. These base lines by disease and by region allows detecting deviations to the established range limits for the sanitary plan established.

INTRODUCTION

Serological evaluation and follow-up is part of a regular health program in the poultry industry. Similar vaccination programs, vaccination techniques, testing methodology, sampling time, genetic line, feeding programs and management systems with the type of production may affect the serological values obtained for the evaluation of an immune response. Serological data on the four most common diseases (Newcastle disease, infectious bronchitis, infectious bursal disease and chicken infectious anemia) were analyzed in this study.

MATERIALS AND METHODS

Location. Three different poultry producing regions in Mexico.

Birds. Broiler breeders of two genetic lines: Cobb 500 and Ross 308.

Vaccination program. A similar vaccination program was administered to broiler breeder flocks in the three regions analyzed.

Diseases monitored. Newcastle disease, infectious bronchitis, infectious bursal disease, and chicken infectious anemia.

Sampling schedule. See Figure 5.

Serological test. Hemagglutination inhibition (HI) test and Idexx ELISA kits.

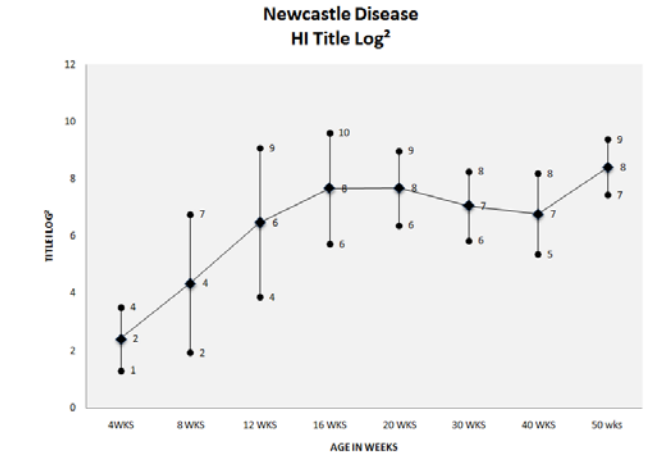
Statistical analysis. Data base represented the arithmetic mean titer with 2 SD +/-.

CONCLUSIONS

The broiler breeders received a total 11 Newcastle disease live conventional vaccinations, two inactivated vaccines and a vector rHVT-F recombinant vaccine. The serological values for Newcastle disease by HI ranged between seven and eight $\log_2$ after 20 weeks of age with a coefficient of variation ≤ 20.

The arithmetic mean titers for infectious bronchitis by the Idexx ELISA kit ranged between 5,000 and 6,500 with a coefficient of variation $\leq 48\%$. Birds received a total of five live and two inactivated vaccines. The arithmetic mean titers for infectious bursal disease using a conventional Idexx ELISA kit ranged between 6,000 and 7,500 with a coefficient of variation of ≤ 40. Breeder received a total of two live and two inactivated vaccines. Finally, the corresponding arithmetic mean titer for chicken infectious anemia ranged between 9,000 and 10,000 with a coefficient of variation of $\leq 38\%$ and received only one live vaccination at around 12 weeks of age.

Figure 1. Newcastle disease blood sampling at eight different ages using HI with four hemagglutinating units.
Figure 2. Infectious bronchitis blood sampling at eight different ages using the IB ELISA kit from Idexx.



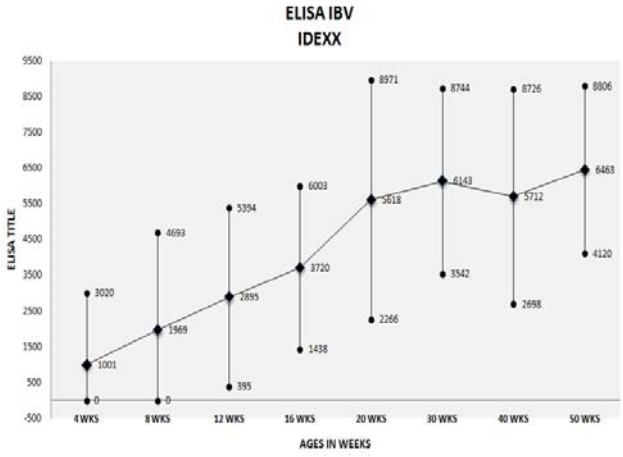


Figure 3. Infectious bursal disease blood sampling at 10 different ages and using the Idexx ELISA kit.
Figure 4. Chicken infectious anemia with three blood samplings at different ages and using an Idexx ELISA kit.

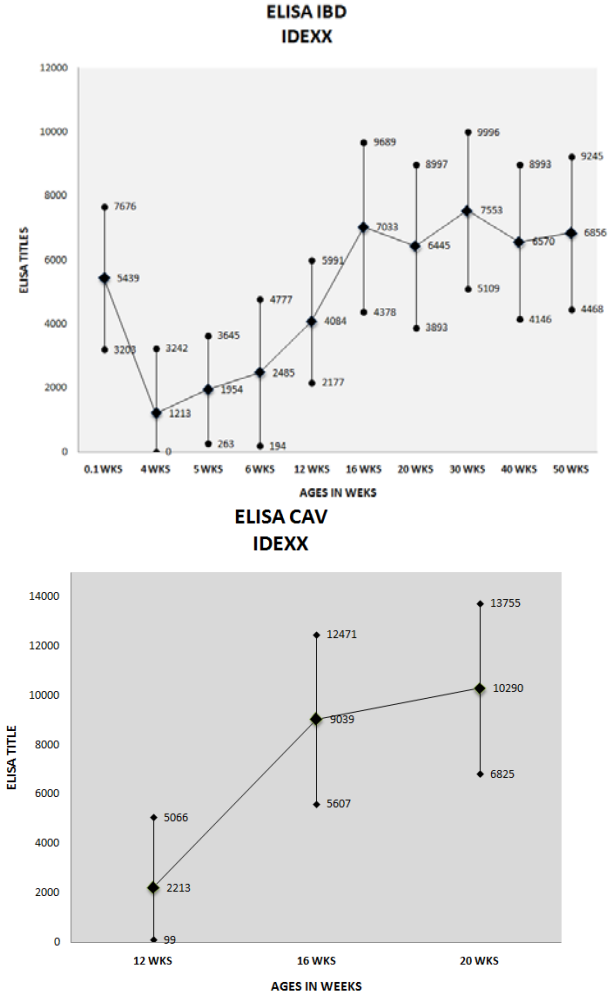


Figure 5.

Sampling Dates	Weeks of Age										
	0.1	4	5	6	8	12	16	20	30	40	50
NDV		●			●	●	●	●	●	●	●
IBV		●			●	●	●	●	●	●	●
IBD	●	●	●	●		●	●	●	●	●	●
CAV						●	●	●			

RESULTS OF INFECTION WITH *AVIBACTERIUM PARAGALLINARUM* AND/OR *GALLIBACTERIUM ANATIS* IN SPECIFIC-PATHOGEN-FREE CHICKENS AND THE ROLE OF VACCINATION IN PREVENTING DISEASE

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SUMMARY

Avibacterium paragallinarum (1,5) and *Gallibacterium anatis* (2) are recognized bacterial pathogens both infecting the respiratory tract of chickens (4). It has been reported that concomitant infection with other pathogens (*Mycoplasma gallisepticum* and *Ornithobacterium rhinotracheale*) can intensify the clinical signs and pathological lesions of coryza cases reported from field outbreaks and experimental infections (3,8). The present study investigated outcomes of an infection with *Avibacterium paragallinarum* and/or *Gallibacterium anatis* in specific-pathogen-free chickens by elucidating clinical signs, pathological lesions and bacteriological findings. Additionally, the efficacy of a commercially available vaccine to prevent diseases caused by *Av. paragallinarum* and *G. anatis* was evaluated. Birds inoculated with *G. anatis* alone did not present any clinical signs and gross pathological lesions in the respiratory tract. However, clinical signs of infectious coryza were reproduced in non-vaccinated birds that were challenged with *Av. paragallinarum* alone or together with *G. anatis*. Such clinical signs were more severe in the co-infected group including mortality. Some of the birds which were vaccinated and challenged showed mild clinical signs at seven days post infection (dpi). Inflammation of sinus infraorbitalis was the most prominent gross pathological lesion found in the respiratory tract of non-vaccinated birds inoculated either with *Av. paragallinarum* and *G. anatis* or *Av. paragallinarum* alone. In the reproductive tract, haemorrhagic follicles were observed in non-vaccinated birds that were infected either with *G. anatis* (6,7) alone or together with *Av. paragallinarum*. In vaccinated birds, no gross pathological lesions were found except in one bird which was co-infected with both the pathogens characterized by mucoid tracheitis. Bacteriological investigations revealed that multiplication of *G. anatis* at seven dpi was supported by the co-infection with *Av. paragallinarum*. Altogether, it can be concluded that simultaneous infection of *Av. paragallinarum* and *G. anatis* can increase the severities of disease conditions in chickens. In such a scenario, vaccination appears to be an effective tool for prevention of the disease as protection was conferred based on clinical, pathological, bacteriological and serological data.

REFERENCES

1. Blackall, P. J., Christensen, T. Beckenham, L. L. Blackall, and M. Bisgaard. Reclassification of Pasteurella gallinarum, [Haemophilus] paragallinarum, Pasteurella avium and Pasteurella volantium as Avibacterium gallinarum gen. nov., comb. nov., Avibacterium paragallinarum comb. nov., Avibacterium avium comb. nov. and Avibacterium volantium comb. nov. Int. J. Syst. Evol. Microbiol. 55:353-362. 2005.

2. Christensen H., M. Bisgaard, A. M. Bojesen, R. Mutters, and J. E. Olsen. Genetic relationships among avian isolates classified as Pasteurella haemolytica, 'Actinobacillus salpingitidis' or Pasteurella anatis with proposal of Gallibacterium anatis gen. nov., comb. nov. and description of additional genomospecies within Gallibacterium gen. nov. Int. J. Syst. Evol. Microbiol. 53:275-287. 2003.

3. Morales-Erasto V., F. Falconi-Agapito, G. A. Luna-Galaz, L. E. Saravia, A. Montalvan-Avalos, E. E. Soriano-Vargas, and M. Fernandez-Diaz. Coinfection of Avibacterium paragallinarum and Ornithobacterium rhinotracheale in Chickens from Peru. Avian Dis. 60:75-78. 2016.

4. Neubauer C., M. De Souza-Pilz, A. M. Bojesen, M. Bisgaard M, and M. Hess. Tissue distribution of haemolytic Gallibacterium anatis isolates in laying birds with reproductive disorders. Avian Pathol. 38:1-7. 2009.

5. Page L. A. Haemophilus infections in chickens. I. Characteristics of 12 Haemophilus isolates recovered from diseased chickens. Am J. Vet. Res. 23:85-95. 1962.

6. Paudel S., D. Liebhart, C. Aurich, M. Hess, C. Hess. Pathogenesis of Gallibacterium anatis in a natural infection model fulfils Koch's postulates: 2. Epididymitis and decreased semen quality are the

predominant effects in specific pathogen free cockerels. Avian Pathol. 43:529-534. 2014.

7. Paudel S., D. Liebhart, M. Hess, and C. Hess. Pathogenesis of Gallibacterium anatis in a natural infection model fulfils Koch's postulates: 1. Folliculitis and drop in egg production are the predominant effects in specific pathogen free layers. Avian Pathol. 43:443-449. 2014.

8. Sandoval V. E., H. R. Terzolo, and P. J. Blackall. Complicated infectious coryza outbreaks in Argentina. Avian Dis. 38:672-678. 1994.

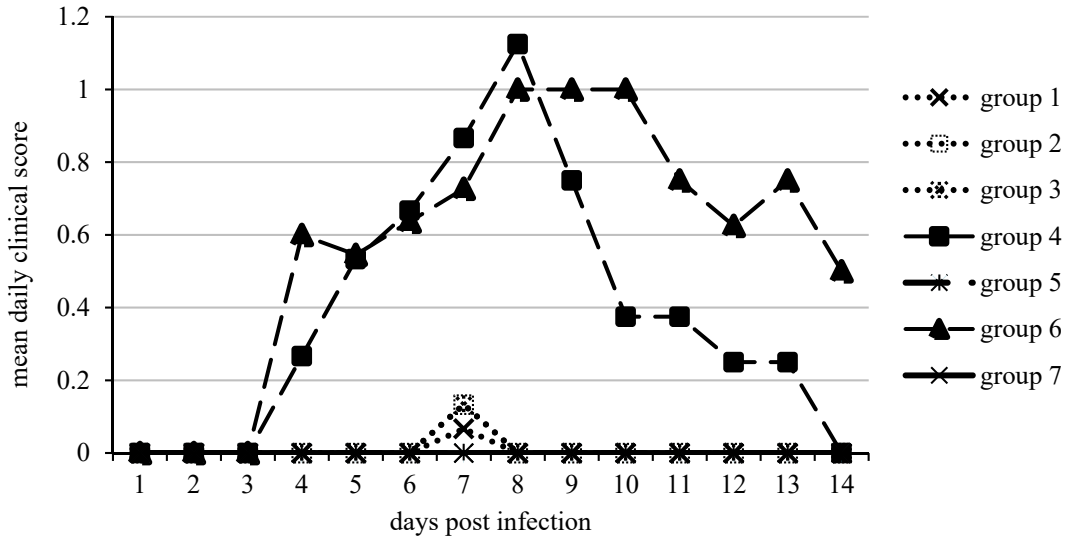
(A full report of these studies has been submitted to *Avian Diseases*.)

Table 1. Experimental design.

Group	No. of birds	Vaccination (week of life)		Challenge (week of life)	Necropsy (dpi)[A]	
		6[B]	10[C]	14	7	14
1	15	yes	yes	*Av. paragallinarum*	7[D]	8
2	15	yes	yes	*G. anatis*	7	8
3	15	yes	yes	*Av. paragallinarum* + *G. anatis*	7	8
4	15	no	no	*Av. paragallinarum*	7	8
5	15	no	no	*G. anatis*	7	8
6	15	no	no	*Av. paragallinarum* + *G. anatis*	3[E]	8
7	15	no	no	no	7	8

[A] dpi: days post infection; [B] first vaccination; [C] second vaccination with a combined killed vaccine: *Avibacterium paragallinarum* and *Gallibacterium anatis* (Volvac® Gallibacterium + AC Plus KV, Boehringer Ingelheim); [D] number of birds for necropsy; [E] as four birds died at 5 dpi only three birds were left for necropsy examination at 7 dpi.

Figure 1. Mean daily clinical score of birds in different groups (observation period until 14 dpi).



Scoring system: 0-no signs; 1-mild nasal discharge or mild facial edema; 2-nasal discharge with facial edema; 3-severe facial edema with or without conjunctivitis, birds showing apathy

MODELING WATERFOWL HABITAT SELECTION IN THE CENTRAL VALLEY OF CALIFORNIA TO BETTER UNDERSTAND THE SPATIAL RELATIONSHIP BETWEEN COMMERCIAL POULTRY AND WATERFOWL

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ABSTRACT

Wildlife researchers frequently study resource and habitat selection of wildlife to understand their potential habitat requirements and to conserve their populations. Understanding wildlife spatial-temporal distributions related to habitat have other applications such as to model interfaces between wildlife and domestic food animals in order to mitigate disease transmission to food animals. The highly pathogenic avian influenza (HPAI) virus represents a significant risk to the poultry industry. The Central Valley of California offers a unique geographical confluence of commercial poultry and wild waterfowl, which are thought to be a key reservoir of avian influenza (AI). Therefore, understanding spatio-temporal distributions of waterfowl could improve our understanding of potential risk of HPAI exposure from a commercial poultry perspective. Using existing radio-telemetry data on waterfowl (U.S. Geological Survey) in combination with habitat and vegetation data based on Geographic Information Systems (GIS), we are developing GIS-based statistical models that predict the probability of waterfowl presence (Habitat Suitability Mapping). Near-real-time application can be developed using recent habitat data derived from Landsat imagery (acquired by satellites and publically available through the U.S. Geological Survey) to predict temporally- and spatially-varying distributions of waterfowl in the Central Valley. These results could be used to provide decision support for the poultry industry in addressing potential risk of HPAI exposure related to waterfowl proximity.

INTRODUCTION

Wildlife researchers frequently study resource and habitat selection of wildlife to understand their potential habitat requirements and to conserve their populations. Despite loss of more than 90 percent of its historic wetlands, the Central Valley of California (Fig. 1) is one of the most important wintering regions for migratory waterfowl (ducks, geese, and swans) in North America, supporting approximately 60 percent of the Pacific Flyway waterfowl population and 18 percent of its continental population (1, 2). Wetlands and certain agricultural lands in the valley provide critical winter roosting and feeding habitat, where waterfowl can replenish energy reserves depleted during fall migration, survive the winter, and ultimately improve body condition for spring migration and breeding (3). Important cropland habitats for waterfowl in the valley include rice and corn fields that are left unplowed and rice, corn, and other crop fields flooded after harvest (4).

Understanding spatial-temporal waterfowl distributions related to habitat is essential for understanding the spatio-temporal relationship between waterfowl and commercial poultry. In 2014-2015, an outbreak of HPAI occurred in commercial poultry in North America which led to the depopulation of over 45 million poultry at approximately 200 operations in 15 states, including California, with a total economic impact over $1 billion (5). In response, at least 29 countries as well as the European Union banned importation of poultry from affected states.

Wild waterfowl are potentially key reservoirs of the virus, and understanding their role in transmission and spread of HPAI is critical in preventing further outbreaks. One approach toward mitigating risk involves better understanding of spatio-temporal epidemiology of HPAI-infected waterfowl. Therefore, understanding spatio-temporal distributions of waterfowl could improve our understanding of potential risk of HPAI exposure from a commercial poultry perspective.

Although waterfowl habitats in the Central Valley generally are well understood, detailed analyses are required to understand response of waterfowl distributions to management- and weather-related changes in these habitats as well as from

disturbance caused by hunting. Such a detailed analysis would not only provide useful information for conservation of waterfowl in the Central Valley, but also promote understanding of spatio-temporal risk of HPAI exposure for greater protection of California's poultry industry. The U.S. Geological Survey has established long-term research on infectious diseases, birds, watersheds and environmental conditions and a science strategy to significantly contribute to the understanding of AI and provide guidance for decisions made by resource managers and policymakers (6).

The objective of this research is to evaluate spatio-temporal distributions of wintering migratory waterfowl based on a representative suite of Central Valley waterfowl (green-winged teal, mallard, northern pintail, and greater white-fronted goose), in relation to habitat management, weather, and hunting that affect waterfowl use of roosting habitats. A major goal of the research team is to integrate results from this analysis with other research conducted by the team to develop a spatially- and temporally-explicit HPAI-risk "early warning" system that will be accessible on the University of California Cooperative Extension (UCCE) poultry website. Other research conducted by the team also includes using "next-generation" weather radar (NEXRAD) to detect waterfowl and map their distributions. Although NEXRAD provides accurate and spatially-explicit information on waterfowl distributions, NEXRAD does not provide complete spatial coverage of the Central Valley, nor waterfowl distributions in the valley. Models developed based on historical waterfowl radio-telemetry data will be used to predict waterfowl distributions outside of NEXRAD coverage.

METHODS

Study area. The Central Valley encompasses about 52,000 km^2 between the city of Red Bluff in the north and the city of Bakersfield in the south, and east-west between the foothills of the Sierra Nevada and Pacific Coastal Ranges (2; Fig. 1a). The Sacramento Valley in the northern Central Valley is the main rice-growing region in the state, but rice and corn fields are also important waterfowl habitats in the Sacramento-San Joaquin River Delta (4). Most post-harvest flooding of other crops occurs in the Tulare Lake bed, in the southern part of the Central Valley (7). The Central Valley is generally characterized as having a Mediterranean-type climate with warm, dry summers and cool, wet winters (2). Most surface water supplies used in CVCA, including for waterfowl habitats, are produced by snowmelt and rainfall draining into storage reservoirs, and groundwater is extensively pumped when surface supplies are unavailable.

Analytical approach. Using existing radio-telemetry data on waterfowl (USGS) in combination with habitat and vegetation data based on GIS, we are developing GIS-based statistical models that predict the probability of waterfowl presence. We are developing the models based on available waterfowl radio-telemetry data (n = 696 ducks and 120 geese, point locations recorded at one to two day intervals for each bird; years 1987–2000, non-breeding period, in Central Valley, Fleskes unpubl. data) and best available land cover, habitat management, and surface water flooding (GIS) data representing monthly time intervals. Explanatory variables calculated from these habitat data will be modeled by comparing habitat units used by radio-marked waterfowl during day-time hours (considered roosting) with randomly-selected habitat units. We will use modeling results to estimate and map the probability of waterfowl at each available habitat unit. Modeling results on waterfowl use of habitat units will be compared with independent data on distributions of waterfowl detected using NEXRAD to assist with selecting and validating models. Ultimately, selected models will be used to predict waterfowl roosting locations extending beyond the area that NEXRAD can effectively be used to detect waterfowl. This project is on-going and modeling results are not completed. Results that are presented are preliminary, do not include analysis for white-fronted goose, and are likely to change during the course of the study. Figure 1b and Figure 1c represents the density of telemetry locations of ducks (multiple locations/duck) between 1997 and 2000. Herein, we provide preliminary results on relationships between locations of radio-marked ducks and land cover, and radio-marked duck locations and locations of commercial poultry facilities (data not shown in Fig. 1).

RESULTS

Radio-telemetry data (years 1997–2000) for the three duck species evaluated generally corresponds with areas of rice fields and wetlands in the Sacramento Valley and Suisun Marsh, areas of corn in the Delta, and areas of wetlands in the San Joaquin Valley (Fig. 1). Kernel density estimates suggest that the spatial distribution of ducks, based on analysis of the three representative species, generally did not closely coincide with locations of commercial poultry facilities during years 1997–2000. However, in several instances, high densities of duck point locations were relatively near commercial poultry facilities, particularly in the San Joaquin Valley.

CONCLUSION

Some poultry facilities appeared to be at relatively high risk of exposure to HPAI from waterfowl based on historical telemetry data for radio-marked ducks. Facilities located in the San Joaquin Valley appeared to be at relatively higher risk, whereas those outside of the Central Valley appear to be at least risk of exposure to HPAI. Although preliminary, these results illustrate the potential for using historical telemetry data combined with geo-spatial habitat data to help model and identify areas (especially outside of NEXRAD coverage) for prioritizing more intensive biomonitoring and biosecurity measures to reduce potential risk of HPAI transmission to poultry.

REFERENCES

1. Gilmer, D.S., M.R. Miller, R.D. Bauer, and J.R. LeDonne. California's Central Valley wintering waterfowl: concerns and challenges. Trans. N. Am. Wildl. and Nat. Resour. Conf. 47:441–452. 1982.

2. Fleskes, J.P. Wetlands of the Central Valley of California and Klamath Basin. In: Wetland habitats of North America: ecology and conservation concerns. D. Batzer and A. Baldwin, eds. University of California Press, Berkeley. pp. 357–370. 2012.

3. Fleskes, J.P., J.L. Yee, G.S. Yarris, D.L. Loughman. Increased body mass of ducks wintering in California's Central Valley. J. Wildl. Manage. 80:679–690. 2016.

4. Central Valley Joint Venture. Central Valley Joint Venture Implementation Plan: conserving bird habitat. Report. Sacramento, CA. U.S. Fish and Wildlife Service. Available from: http://www.centralvalleyjointventure.org/assets/pdf/CVJV_fnl.pdf. 2006.

5. Eller, D. Bird flu shows U.S. not ready for next big outbreak. Des Moines Register. 1 June 2015. http://www.desmoinesregister.com/story/money/agriculture/2015/05/30/bird-flu-iowa-minnesota-avian-influenza-outbreak-biosecurity-usda/28217131/. 2015.

6. Harris, M.C., A. K. Miles, J. M. Pearce, D. J. Prosser, J. M. Sleeman, and M. E. Whalen. USGS highly pathogenic avian influenza research strategy: U.S. Geological Survey Fact Sheet 2015-3060, http://dx.doi.org/10.3133/fs20153060. 2015. (Accessed 13 Apr. 2016).

7. Fleskes, J.P., D.A. Skalos, M.A. Farinha. Changes in types and area of postharvest flooded fields available to waterbirds in the Tulare Basin, California. J. Fish Wildl. Manage. 4:351–361. 2013.

Figure 1a. Waterfowl habitats in the Central Valley of California (corn displayed for the southern Central Valley is assumed to be unavailable as habitat because of post-harvest practices).

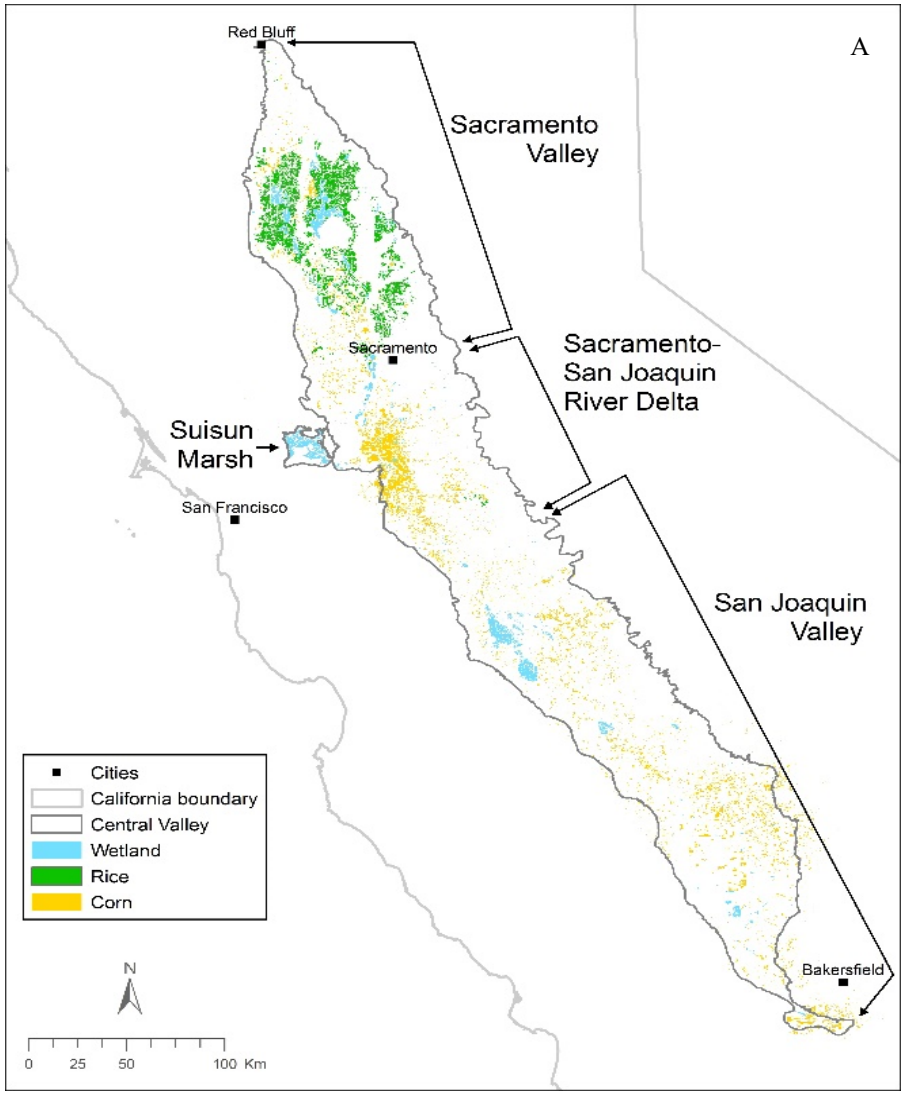


Figure 1b. Point locations of radio-marked green-winged teal, mallards, and northern pintails in years 1997–2000.

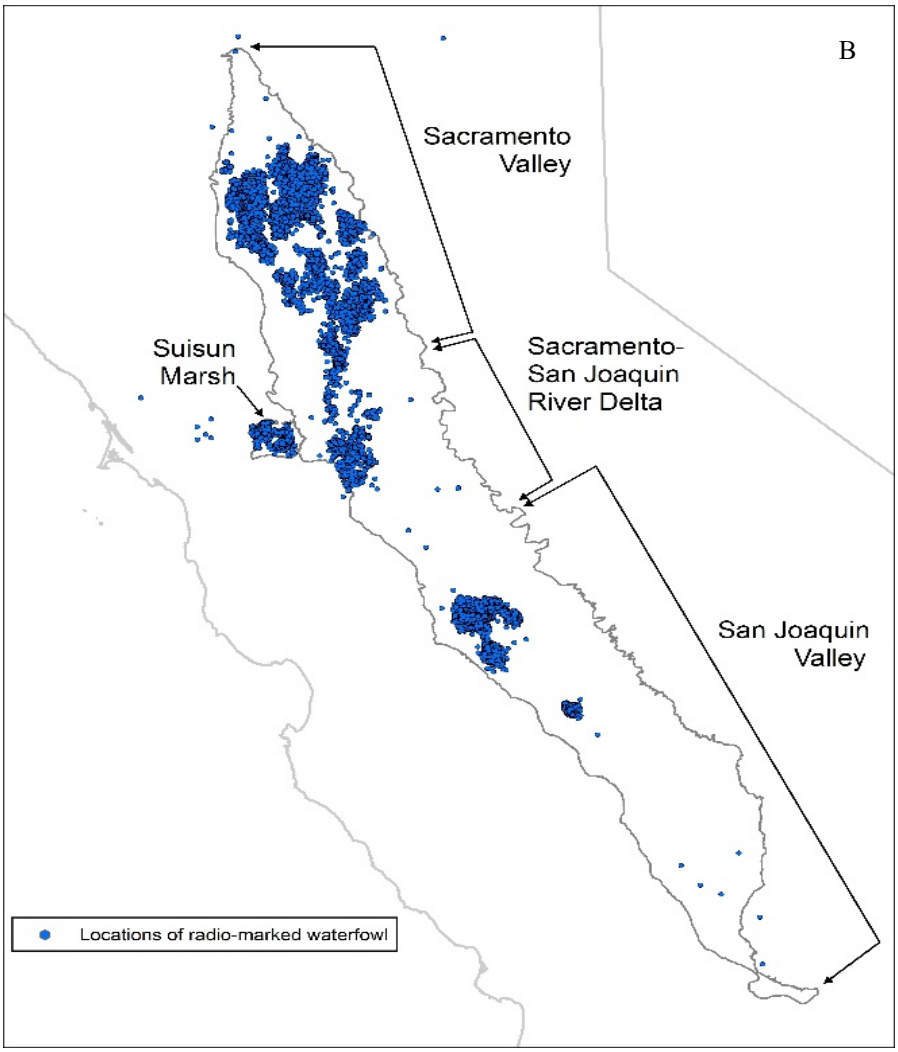
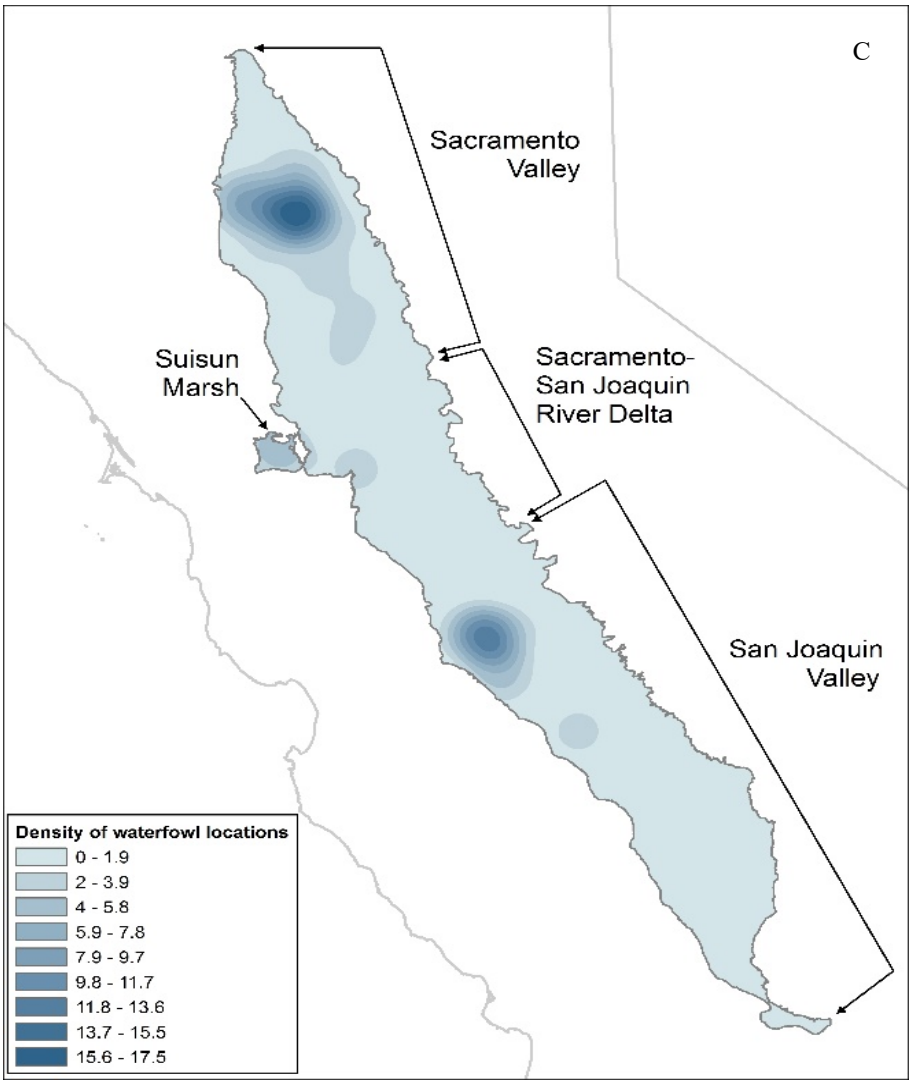


Figure 1c. Kernel density of point locations displayed in B.



THE IMPACT OF WATER OR GEL DAY OF AGE SPRAY ON CHICK CLOACAL TEMPERATURE

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SUMMARY

Successful mass application is critical to the success of any broiler coccidiosis vaccination program. All live coccidiosis vaccines require oral ingestion. A previous study done at the University of Delaware's Lasher Lab showed that the spray cabinet efficacy is approximately 88 percent based on oocyst shed rates five to eight days post hatch. The question remains can we improve beyond this 88 percent? Using the following paper as a starting point (Caldwell *et al*. (2001) *Journal of Applied Poultry Research* 10: 107 – 111) research was implemented to determine if increasing relative photo intensity at the time of spray application can have a positive impact on vaccine uptake; it did. The following year we looked at volume and how it impacted occyst shed based on oocyst per gram of feces. The impact was positive as well. The main concern that was expressed with the increase volume was the impact on chick core temperature. A controlled study was done to determine the impact of the increased volume on chick cloacal temperatures. Gel versus water application was evaluated in both summer and winter settings. The findings from this study will be presented.

THE IMPACT OF A COCCIDIOSIS VACCINE FIELD BOOST

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SUMMARY

As has been stated multiple times, successful mass application is critical to the success of any broiler coccidiosis vaccination program, and all live coccidiosis vaccines require oral ingestion. Starting in 2011, Merial Select began to investigate options to assure the success of mass application. Using the following paper as a starting point (Caldwell *et al*. (2001) *Journal of Applied Poultry Research* 10: 107 – 111) research was implemented to determine if increasing relative photo intensity at the time of spray application has a positive impact on vaccine uptake; it does. We then looked at volume and how it impacted occyst shed based on oocyst per gram of feces. It too has a very positive effect.

Now Merial Select believes the next opportunity is in the chicken house. With the industry moving away from many traditional ways to manage cocci and related disease challenges in the chicken house, Merial Select has been investigating the impact of field boosting a coccidiosis vaccine. Over the past three years several studies have been done and the results are very encouraging.

Data will be presented that show the effect on oocyst shed patterns, lesion scores from challenge models, and actual field data from production companies that have used this tool.

IGA QUANTITATION IN DIFFERENT BIOLOGIC SAMPLES FROM BROILER CHICKENS VACCINATED AGAINST NEWCASTLE DISEASE AND INFECTIOUS BRONCHITIS

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In poultry, several vaccines are given as live antigens, by either, the nasal, oral, or conjunctival routes, allowing the mucosa-associated lymphoid tissue, through the plasma B cells, to produce large quantities of secretory immunoglobulin A (SIgA), which is the most important immunoglobulin in external secretions. Many studies have been developed to detect SIgA after vaccinating the birds; however, detection of specific IgA usually is done by ELISA tests measuring the optical density (OD) in the samples.

In this study, an IgA positive reference sample was produced first and then used to titer anti Newcastle disease (ND) and anti infectious bronchitis (IB) IgA in different biological samples from broiler chickens. First, white Leghorn type chickens were vaccinated against ND and IB at 14, 30 and 51 days of age. Samples of lachrymal fluid, cloacal swab and saliva were tested at different ages by a commercial ELISA test (Affinitech, LTD, AR, USA), which anti chicken IgG conjugate was replaced by an anti chicken IgA (Bethyl Lab., TX, USA).

Two ways to determine positive samples to anti NDV IgA or anti IBV IgA were used:

1) OD comparison between pre- and post-vaccination samples, by ANOVA.

2) Positive sample threshold: mean optical density value from pre-vaccination samples was calculated, and three times the value of its standard deviation (SD) was added to that value, samples with an OD value higher than the threshold were considered as positive. In the second part, broiler chickens were used.

All birds were vaccinated at seven days of age by the ocular route with a combined vaccine ND-BI, live virus. At 21 days of age, lachrymal fluid, tracheal and cloacal swab samples were obtained and tested by ELISA.

Besides the ways used in Experiment 1, the samples having the highest OD for either, NDV or IBV from the samples tested in Experiment 1 were used as positive control to calculate the sample to positive ratio (S/P) of all broiler chicken samples. An S/P threshold of 0.15 was established to identify positive samples.

ELISA titers: Samples having an S/P ratio lower than the threshold received an IgA titer of 0, samples with S/P ratio higher than the threshold were used to estimate the relative IgA titer using the UniVET software provided by Affinitech, with the same parameters used to calculate IgG titers.

In broiler chickens, specific IgA against either ND and BI was detected more in lachrymal fluid samples than in tracheal samples. Cloacal samples were negative to IgA. The S/P ratio allowed to detect more positive samples in ND and IB vaccinated chickens than those detected by the OD threshold of unvaccinated samples plus three SD. ELISA titers calculated with the UniVET software were higher in IB vaccinated chickens than in ND vaccinated ones.

These results show that getting a positive chicken IgA anti ND or anti IB reference sample is useful to better detect and quantify specific IgA against several antigens. With this protocol, the humoral local response measurement can be easily established in any serology laboratory to measure the local immune response stimulated by live vaccines.

(The full-length article will be published in *Journal of Veterinary Diagnostic Investigation*.)

MAREK'S DISEASE IN BACKYARD CHICKENS, A STUDY OF PATHOLOGICAL FINDINGS AND VIRAL LOADS IN TUMOROUS AND NON-TUMOROUS BIRDS

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SUMMARY

Marek's disease (MD) is a major cause of mortality in backyard chickens. The diagnosis of MD is complex, however, and knowledge on Marek's disease virus (MDV) in spontaneous field cases such as in backyard chickens is largely unknown. In this study, 40 backyard chickens with presumptive MD diagnosis based on histological lymphoid infiltrations in peripheral nerves with and without lymphomas were investigated. Twenty-eight of the birds were submitted to the diagnostic laboratory for disease explorations, and 12 chickens were from a flock where some demonstrated anisocoria and pupil irregularities compatible with ocular MD. Histological scores were established for brain, peripheral nerves, heart, lung, liver, kidney and gonad sections, ranging from mild (+) to severe (+++) lymphoid infiltrations. Twelve chickens had gross lymphomas, all but two chickens had mild to severe peripheral nerve lymphoid infiltrates. There were no age or breed predispositions in the study group. Quantification of serotypes MDV-1, 2 and 3 performed using real-time PCR demonstrated high correlation (R^2 = 0.94) between fresh and fixed spleen specimens, as well as between histopathology scores and MDV-1 viral loads. MDV-2 DNA was detected in a portion of the chickens, likely consistent with naturally occurring virus, whereas the vaccine strain MDV-3 was rarely detected. Significant differences in MDV-1 viral loads between tumorous and non-tumorous chickens were observed, where a ratio of MDV-1 gB/GAPDH $\geq$0.5 was suggestive of gross tumors in this study. We propose that real-time PCR may be a good tool for MD diagnosis in backyard chickens.

(The full-length paper is published in *Avian Diseases* 60: 826-836, 2016.)

CHALLENGES IN NUTRITION OF ORGANIC POULTRY FLOCKS

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SUMMARY

The purpose and goals of organic poultry production have now been well defined and seem, at times, contradictory to the regulations and guidelines established for this industry. In principle, organic farming has been interpreted as an approach to agriculture where the aim is to create integrated, humane, environmentally and economically sustainable agricultural production systems leading to the production of acceptable levels of crop, poultry, and human nutrition (8). Organic farming standards were originally designed as a holistic approach to reducing the impact of food production in an environment that concentrates on animal living conditions that promote natural behavior and emphasizes preventive health management without the use of synthetic or unnatural products (11). This production is meant to specifically avoid synthetic pesticides, fertilizers, growth promoters and other antibiotics, and it focuses on animal health and welfare, good environmental practices, and optimum production quality, with much less emphasis on maximizing production or profits (11).

Although consumers often link organic farming with small scale production (3), today's organic poultry production is dominated by the same large scale producers that provide most of the commercial meat in conventional markets. In the last few years, California, Pennsylvania, and Nebraska companies have become the biggest producers of organic birds (3). As a consequence, organic poultry production not only faces the same issues associated with conventional production including breed sourcing and rearing of stock, housing, nutrition, animal health, and slaughter and processing facilities (8), but additional unique requirements associated with organic regulations. Thus, organic production has evolved as a branch of commercial poultry production that may not necessarily fulfill producers, consumers, or animal health and welfare expectations (3).

As the organic certification program developed, quality control of organic products has shifted from the original producer to government control and certifying organizations (3). Feed formulation and manufacturing of organic feeds are regulated by the United States Department of Agriculture (USDA) National Organic Program (NOP) (4). In general, organic production standards are more comprehensive and restrictive than the standards for conventional poultry production (8). The NOP standards, however, are general guidelines in that details are not specified by the NOP. Rather, details are commonly interpreted by certifying agencies accredited by the NOP (11). So while the NOP national list specifies which ingredients are allowed in organic feeds (11), the specific production requirements for organic production vary somewhat depending on the certifier.

There are unique challenges in organic poultry feed production. As a general rule, organic ingredients cannot be chemically treated (11). Animal drugs such as antibiotics (12), animal by-products, and genetically modified organisms (GMOs) are theoretically not allowed (11). This last statement is not entirely correct. For grains to be certified organic the original planting seed must be GMO free and the crop producer must exercise reasonable precautions to maintain the crop GMO free. Finding GMO cross-contamination in organic grains, however, does not decertify an organic crop. Synthetic vitamins and conventional minerals are allowed but must comply with the Federal Food, Drug and Cosmetic Act (11). Synthetic amino acids are not permitted but methionine use has been temporarily waived at limited levels. Some exogenous enzymes and inorganic sources of calcium and phosphorus are allowed. Synthetic antioxidants such as ethoxyquin cannot be supplemented (11).

Perhaps the biggest challenge in organic feed production is the limited used of synthetic methionine (4). As of October 2, 2012, organic turkey feeds in the United States may contain no more than three pounds of synthetic methionine per ton whereas all other poultry diets may contain no more than two pounds per ton. These methionine restrictions are not sufficient to meet optimal methionine practical requirements. Methionine is an essential amino acid. It is required for protein synthesis and as a methyl donor for several compounds, including choline, creatine, epinephrine, DNA, and glutathionine (7). Methionine also plays an important role in the support of connective tissue and immunity. A deficiency causes reduced feed intake and growth and, more importantly, a deficiency leads to feather pecking and eating and cannibalism. Feather pecking causes reduced feather cover, and it exposes the skin to injury and impaired thermoregulation (7).

Meeting of current nutrient requirements for optimum poultry production without synthetic amino acids has proven to be impossible, particularly during the first seven days of development of broilers and turkeys, where body weight increases approximately 400 percent (2). Nutrient requirements for birds grown organically is the same as for conventional birds, but balanced organic diets are difficult to produce because of current constraints in organic feeds. In order to meet these requirements, nutritionist either feed more digestible ingredients or use greater than normal levels of nutrients from main organic ingredients (2). Because there is no known natural organic ingredients with levels of methionine high enough to compensate for the lack of synthetic methionine availability (4), nutritionists attempt to meet methionine requirements by increasing dietary levels of organic soybean meal and end up overfeeding protein. It has been estimated that in order to fulfill the sulfur amino acid requirements in organic poultry diets, levels of other amino acids need to be increased by 13 to 31 percent above optimal requirements (10). Higher than optimal protein levels have a negative effect on gut health and will cause stress (10). As birds deal with excess nitrogen metabolism, it creates a burden on the kidneys, increases microbial growth, increases manure moisture, creates poor litter quality, and increases ammonia production, which leads to breast blisters and footpad lesions as well as unnecessary increases in feed cost (7). There is a linear relationship between dietary crude protein levels and ammonia emission from poultry litter (9). Increases in ammonia production in the growout house is also detrimental to poultry production personnel (4). Because of current organic regulations, nitrogen and sulfur pollution of air and water due to organic production has also been exacerbated. Nutrition strategies used in conventional poultry production to reduce nutrient excretion; a reduction of excess dietary nutrients cannot be easily implemented in organic poultry production (9).

Because conventional, fast growing chicks are used in organic production, the apparent solution would be to switch to a slower growing breed that has lower sulfur amino acid requirements. Recent data, however, indicates slow growing breeds do not have lower methionine requirements (11). This suggests the requirements for methionine for fast and slow growing birds is the same, but faster growing breeds are more efficient at methionine metabolism.

While current regulations prohibit the routine use of antibiotics and parasiticides in organic poultry production, the need for these products may be more critical that in conventional bird production. It is important to point out that current regulations indicate antibiotics and other medical treatments must not be withheld in organic poultry if they are needed, but birds treated with prohibited materials cannot be marketed as organic (11). Environments in which birds are exposed to soil provide a greater opportunity for disease and parasites as these birds are in direct contact with soil borne bacteria and parasites and have a greater risk of infectious disease exposure from wild animals compared to birds grown under conventional practices (5). In fact, outdoor requirements for organic poultry potentially increase exposure to bacteria such as *Salmonella*. Recent data suggests organic chicken tends to have a higher incidence of *Salmonella* than conventional birds (12). Thus, while elimination of antibiotics and other veterinarian medicinal inputs may tend to improve product quality by decreasing risk of residue and minimizing bacterial resistance, these practices may increase the chances of man-to-animal disease transmission and may cause unnecessary animal suffering from or at risk of disease due to limited access to routine and or preventive medications (3).

Coccidiostat use in organic poultry production is also prohibited, and this creates further problems. Cocci challenges often lead to necrotic enteritis due to opportunistic Clostridium bacterial challenges (2). Coccidial vaccines, when available for organic poultry production, have not completely replaced coccidiostats, as they also quickly lead to resistance (2) Thus, necrotic enteritis is a common health problem in organic flocks (11).

As we look for alternatives to use of antibiotics and coccidiostats in organic poultry production, the industry has turned to an increase in vaccine use. An immune response, however, has a physiological cost. The limited and transient morbidity associated with a live vaccine for coccidiosis and respiratory diseases leads to significant detrimental secondary systemic responses associated with the inflammatory response including fever, anorexia, carbohydrate storage depletion, protein destruction, and sleep deprivation (6). Inflammation of the gut due to subclinical coccidiosis or necrotic enteritis remains a major animal welfare problem.

The challenge in the production of antibiotic and coccidiostats free birds is how to manage gut micro flora to prevent enteric health issues (7, 13), as the loss of use of these products results in bird health issues and overall loss of performance, without damaging gut dynamics which could also be detrimental to performance (12). This has forced nutritionists to study the interactions between feed ingredients and nutrient levels, and the dynamics of intestinal bacterial populations (2). In the field, management has looked for substitutes including probiotics, prebiotics, organic acids, and plant extracts (1, 9). Direct fed microbials (probiotics) aim to exclude the colonization of

pathogens in the gastrointestinal track (12). As of today, however, there is no single probiotic organism that can effectively protect against all pathogens (1). Prebiotics are non-digestible, short chain carbohydrates called oligosaccharides (12). They are generally either fructooligosaccharides (FOS) – meant to enhance the growth of beneficial gut bacteria by providing nourishment, or manna oligosaccharides (MOS) – carbohydrates that directly bind bacterial receptors involved in intestinal wall cell attachment. Thus, if bacteria cannot attach to the intestinal wall, they cannot theoretically colonize the host. FOS are generally derived from cereals. MOS are produced from yeast cell wall (1, 12). Organic acids inhibit bacterial colonization by changing pH and aim to reduce bacterial challenges. They include proprionic, formic, butyric, acetic, lactic, and caproic. They can be added in the water or feed (1). Plant extracts are generally mixtures of essential oils, including oils from thyme, clove, turmeric, black pepper, cinnamon, garlic, and oregano. Overall, the use of these substitutes has shown limited success, but they have not been shown to completely replace commercial antibiotics nor coccidiostats (1).

Last, the availability of sufficient organically produced ingredients with appropriate quality for optimum productivity under current and proposed organic standards has become a significant issue (2, 8, 10). The United States does not produce enough organically certified ingredients to meet current demand. Approximately 80 percent of the main ingredients corn and soybean meal used in organic diets are imported from India, China, and South America. Traceability of these ingredients is a challenge. Risk of mycotoxin contamination in organic ingredients is equal or greater in organic crops compared to conventional ones (3). Risk from genetically modified organisms cross contamination, while substantially reduced, continues to be an issue (3).

REFERENCES

1. J. P. Griggs and J. P Jacob. Alternatives to Antibiotics for Organic Poultry Production. J. Appl. Poult. Res. 14:750-756. 2005

2. S. Leeson. Predictions for Commercial Poultry Nutrition. J. Appl. Poult. Res. 17:315-322. 2008.

3. M. Hovi, A. Sundrum, and S. Padel. Organic Livestock Farming: Potential and Limitations of Husbandry Practice to Secure Animal health and Welfare and Food Quality. Proceedings of the 2nd SAFO Workshop. 25-27 March 2004, Witzenhausen, Germany. 2004.

4. H. K. Burley, K. E. Anderson, P. H. Patterson, and P. B. Tillman. Formulation Challenges of Organic Poultry Diets with Readily Available Ingredients and Limited Synthetic Methionine. J. Appl. Poult. Res. 2016.

5. C. Lay Jr., R. M. Fulton, P. Y. Hester, D. M. Karcher, J. B. Kjaer, J. A. Mench, B. A. Mullens, R. C. Newberry, C. J. Nicol, N. P. O'Sullivan. Hen Welfare in Different Housing Systems 1. Poult. Sci. 90(1): 278-294. 2011.

6. L. R. Berghman. Immune Responses to Improving Welfare. Poult. Sci. 95(9): 2216-2218. 2016.

7. Y. V. Thaxton, K. D. Christensen, J. A. Mench, E. R. Rumley, C. Daugherty, B. Feingerg, M. Parker, P. Siegel, and G. Scanes. Symposium: Animal Welfare Challenges for Today and Tomorrow. Poult. Sci. 95(9): 2198-2207. 2016.

8. N. Lampkin. 1997. Organic Poultry Production. Welsh Institute of Rural Studies. Prifysgol Cymru. Aberystwyth. The University of Wales.

9. W. Powers, R. Angel. A Review of the Capacity for Nutritional Strategies to Address Environmental Challenges in Poultry Production. Poult. Sci. 87(10): 1929-1938. 2008

10. M. M. Van Krimpen, F. Leenstra, V. Maurer, and M. Bestman. How to fulfill EU Requirements to Feed Organic Laying Hens 100% Organic Ingredients. J. Appl. Poult. Res. 25:129-138. 2016.

11. A. C. Fanatico, C. M. Owens, and J. L. Emmert. Organic Poultry Production in the United States: Broilers. J. Appl. Poult. Res. 18:355-366. 2009.

12. K. G. S. Lilly, L. K. Shires, B. N. West, K. R. Beaman, S. A. Loop, P. J. Turk, G. K. Bissonnnette, and J. S. Moritz. Strategies to Improve Performance and Reduce Preslaughter Salmonella in Organic Broilers. J. Appl. Poult. Res. 20: 313-321. 2011.

13. M. H. Kogut. Impact of Nutrition on the Innate Immune Response to Infection in Poultry. J. Appl. Poult. Res. 111-124. 2009.

INCLUSION BODY HEPATITIS IN MEXICO

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Investigación Aplicada S.A. de C.V.

SUMMARY

Between April and June 2016, outbreaks of inclusion body hepatitis (IBH) were reported in breeding chicken broilers in Mexico. Liver samples were received from four different farms and a liver maceration was performed to carry out virus isolation on monolayers of liver chicken embryo from Specific Pathogens Agents Free and then PCR and sequencing to know the serotype of the causative agent.

Four viruses were isolated in hepatocyte cultures and the results of PCR and sequencing showed that three of them belong to group 1 of adenovirus serotype 8b and one of them to adenovirus serotype 11. These viruses are different from those found in the 1990s when the serotype 4 adenovirus was prevalent. Also, results suggest the presence of immunosuppressive agents such as infectious chicken anemia virus or the virus of infectious bursal disease or some contamination of the food with mycotoxins.

INTRODUCTION

IBH was reported for the first time in Mexico by Antillon *et al*. in 1975 although the causal agent was not identified (1). Subsequently, in 1992, high mortalities were reported in broiler chickens with pictures characteristic of the hydropericardium syndrome (HPS). The virus was isolated in cultures of chicken embryo kidney cells from specific pathogens free embryos and characterized by specific antisera such as adenovirus group I serotype 4 (2). With this virus a commercial vaccine was registered and HPS outbreaks could be controlled since 1993.

In mid-2016 outbreaks of IBH were reported in the central region of the country, mainly in broiler-breeding hens. Samples of livers were received for the isolation and sequencing of the adenoviruses obtained

The objective of the present work was to isolate and characterize an agent involved in outbreaks of IBH by means of PCR and sequencing tests and to determine if it is the same serotype 4 or some other different serotype.

MATERIALS AND METHODS

Isolation. We recieved samples of liver suspect of IBH from four different farms between April and June, 2016. Isolation of the virus was carried out in cultures of chicken embryo hepatocytes from Specific Pathogens Free embryos (ALPES S.A.DE C.V. Mexico) by techniques already described (3). Suspected liver samples were macerated at a ratio of 20 percent with PBS pH 7.2 with five percent of a mixture of antibiotics (PenStrep, Gibco) then centrifuged at 3500 rpm in a refrigerated centrifuge (Allegra X-22 R. Beckman, Coulter) for 15 minutes. Two wells of a six-well (Corning) microplate were infected with 0.5 mL of the supernatant and incubated for 45 minutes at 37°C and then 3 mL of Media 199 (Gibco) was added with five percent fetal bovine serum (CellGro, Corning) other two wells were not infected and were considered as control. Microplate was incubated for other three or four days at 37°C with five percent CO2 and 75 percent of relative humidity.

PCR and sequencing. Viral DNA was extracted from 300 µL of samples using the MagMAX™ Pathogen RNA/DNA Kit (Applied biosystem). The primers used to amplify the Hexon gene were: A: GGC TCC AGT ATT TTC ACA TCG C y Oligo B: AAA GGC GTA CGG AAG TAA GCC. DNA amplifications were carried out in a total volume of 25 µL containing 5 µL viral DNA, 1.25 µL of each primer [10uM] and 1.25 U Taq DNA polymerase recombinant (invitrogen). The following conditions were used for PCR:

A) denaturation, 94°C, for four minutes.

B) denaturation, 94°C, 20 seg; annealing, 58°C, 30 seg; extension, 72°C, for three minutes; 30 cycles followed by a final extension step, 72°C, for seven minutes.

The amplification products were analyzed by electrophoresis in a 1.5 % agarose gel, stained with Syber gold and visualized by UV transillumination. PCR products were purified using DNA Clean & Concentrator kit (Zymo Research). Sequencing was performed using the Applied Biosystems, model 3130xl. The determined sequences were aligned and compared using the Genious 8.1.5 program.

RESULTS

Samples identified as 02/16, 04/16,05/16 and 07/16 showed cytophatic effect on liver hepatocytes from chicken embryo SPF. The effect consisted of cell rounding and presence of syncytia between 48 and 72 hours after infection from first passage. All liver macerated samples were free of bacteria and fungi

demonstrated by a purity test according 9 CFR 113.16. Table 1 shows the identification of the isolates and the serotype found as well as to the group that they belong.

DISCUSSION

There is scientific evidence that adenoviruses can be present in healthy birds without showing any symptoms or alteration of the productive parameters (4). It has also been documented that adenoviruses are generally not primary pathogens and that their presence becomes apparent when birds are immunosuppressed. The only serotype that if considered as a primary pathogen is the four that gives rise to hydropericardium syndrome which is characterized by high mortalities (5). In this work we found the presence of two serotypes 8b and 11 that differ with what was reported in the decade of the 90, where the only serotype 4 was found in Mexico. Unfortunately, the history of the course of the disease was not available and neither knows mortality rates. However, it is interesting to find other serotypes which could be due to the presence of immunosuppressive agents in the farms or to the presence of mycotoxin. More pathogenicity studies of the strains found are needed to know their role as primary or secondary agents and the possible use of vaccine for those affected farms.

CONCLUSION

The presence of adenovirus could be detected by PCR in suspicious cases of IBH isolated primary in liver chicken embryo cultures and identified as adenovirus. This is the first report of the presence of adenovirus serotypes 8 and 11 in Mexico

REFERENCES

1. Antillon,A., and B.Lucio. 1975. Inclusion Body Hepatitis in Mexico. Avian Dis 19:195-197.

2. Morales,G.A. and E.Lucio. 1992. Algunas Caracteristicas de los agentes presentes en el síndrome del hidropericardio en Mexico. Memorias de la XVII Convencion Anual ANECA, Puerto Vallarta, Mexico. Pp 198-201.

3. Villegas, P. Avian Virus Disease Laboratory Manual. University of Georgia, College of Veterinary Medicine.

4. McFerran,J.B. and J.A. Smith. 2000. Avian Adenovirus. Rev.sci.tech.Off.int.Epiz. 19:589-601.

Table 1.

Sample Identification	Scientific identification	Serotype	Group
02/16	FAdV-8b/Mexico/165753/2016	8b	FAdV E
04/16	FAdV-11/México/04/16-ADV/4245/2016	11	FAdV D
05/16	FAdV-8b/Mexico/1609662/2016	8b	FAdV E
07/16	FAdV-8b/Mexico/1611916/2016	8b	FAdV E

THE RAPIDLY CHANGING LANDSCAPE OF POULTRY PEST CONTROL: WHAT LIES AHEAD?

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SUMMARY

Societal pressures, especially in the developed world, are changing the poultry industry rapidly and dramatically. Concerns for animal welfare and enhanced food safety (e.g. the organic movement) are notable examples. Cultural methods such as beak trimming are being modified, and housing changes include the conversion from traditional cages to enriched cages or various cage-free options for egg-laying hens or other categories such as breeders. As these changes occur, we must keep in mind their sizeable impact on parasites in production systems. Housing styles, such as whether or not to allow prolonged ground contact by birds or provide structures such as nest boxes, substantially dictate the type and severity of parasites. Here we briefly discuss impacts of recent and pending changes in poultry production and housing on parasites, with an emphasis on arthropod pests (lice, mites, fleas, ticks, flies, beetles). The European experience since the late 1990's with the red mite, *Dermanyssus gallinae*, provides a particularly useful lesson for Americans, which we ignore at our peril. We then touch on options for future pest control in these modified systems.

INTRODUCTION

The domestic chicken is unquestionably one of the most important food animals in the world (28). Indeed, there may be no other domestic animal that can be raised under such a range of conditions, even on the tiniest plots of land, and still provide food so efficiently. Like the rest of our domestic animals, however, chickens are host to a veritable legion of parasites, both internal and external, which constantly threaten to compromise production efficiency (1, 29, 13).

Here we will focus on two aspects of poultry production, driven by societal concerns, which present a major challenge for parasite management. Simultaneously, however, those same concerns provide a compelling opportunity (and responsibility) to scientists in developing effective parasite control tactics going forward.

First, a fundamental paradigm is changing: that parasites are manageable using a steadily-evolving stream of synthetic pesticides. Many pesticides are being lost due to resistance development (19), and the rate of production of new materials (or new modes of action) is probably not as high as it was several decades ago. Environmental contamination concerns, interest in organic food, and a desire to produce food with as few synthetic chemical inputs as possible are increasingly on people's minds. Also, costs of product registration (or re-registration), development and testing are rising. Much of animal agriculture, including poultry production, simply may not supply a large enough market to justify those costs for a pesticide (especially a target-specific one), from an industry perspective.

Second, animal welfare has worked its way to the front of society's consciousness (3). This is exemplified by Europe's elimination by 2012 of traditional cages (aka battery cages) for egg production and California's recently-implemented (2015) Proposition 2 (10), which required greatly enhanced space for hens. Moving laying hens into traditional cages in the 1940's and 1950's was a key housing change that had both drawbacks and advantages for animal health and welfare; no single housing system is ideal from a welfare perspective (9). Putting hens in cages resulted in increased economic efficiency and reduced or eliminated many serious parasites through several mechanisms. First, separating hens from contact with their feces broke or reduced the fecal-oral transmission cycles of direct parasites. For certain parasites caging birds also tended to limit outbreaks by reducing intimate contact among larger numbers of hosts. Second, traditional cages mostly eliminated off-host hiding places for external parasites such as the red mite *Dermanyssus gallinae*, bed bugs (*Cimex lectularius*) or the soft ticks (*Argas spp.*). Third, cages got birds off the ground. Earthworms or beetles, intermediate hosts for some parasitic nematodes or tapeworms, normally would be consumed by hens foraging on the ground, but those life cycles were broken. Larvae of sticktight fleas (*Echidnophaga gallinacea*) develop in moist soil, eating organic debris and dried chicken blood. Flea adults have evolved to ingest much more blood than

they themselves need; excess blood is excreted from the anus and falls into the soil/nest debris as a key larval food. So fleas also disappeared from traditional cage systems. To be sure, some parasites became worse after this shift to cages. Flies got much worse in accumulated feces and birds could no longer eat them. Permanent ectoparasites such as northern fowl mites (*Ornithonyssus sylviarum*) and lice (such as *Menacanthus stramineus*), which complete the entire life cycle on the bird and spread easily from cage-to-cage, found massive numbers of hosts in close contact and ready for immediate occupation. However, traditional cages are unfriendly to parasites overall.

PARASITES IN CAGE-FREE SYSTEMS

From this point we will focus mostly blood-feeding ectoparasitic arthropods, including the mites *D. gallinae* and *O. sylviarum*, the sticktight flea *E. gallinaceae*, the bedbug *C. lectularius*, soft ticks (*Argas* spp.), as well as the numerous species of chewing lice that call chickens their home.

In the days before traditional cages, the red mite, *D. gallinae*, used to be a key pest (probably the worst mite pest) in American poultry production (2). Based on extensive experience in southern California caged laying hen houses over 34 years, however, one of us (BAM) has never seen *D. gallinae* in traditionally caged layers, although it has been found in local wild sparrow nests (Mullens, unpublished) and southern California backyard chickens (20). The reason(s) it has not been found remain speculative, but we can make a good guess that this is at least partly due to lack of near-host hiding places for caged layers in traditional cages. The mites love cracks and crevices in things like wooden nest boxes or around perches in other housing, including both enriched cages and cage-free systems (26). They hide in crevices by day and come out at night to blood-feed on the hens. They retreat to their lairs after feeding, and people usually would only see them by day if they pulled those cracks apart.

Europe, beginning with the Scandinavians, outlawed traditional cages in 2012 (27). These were replaced by another cage style (enriched cages) or by cage-free configurations. The enriched cages provide more space per bird, but also provide enrichments in the form of nest boxes, scratch pads and perches. The cage-free options include a wide range of enclosed housing (e.g. aviaries), culminating in free-range or pastured conditions where hens have substantial ground contact. All of these housing designs are somewhat more parasite-friendly than the traditional cages (27, 9). After the transition began, almost immediately European producers began to encounter much worse problems with red mites (8). In fact those mites became key pests and have been very hard to manage (15, 26, 27). Besides giving the red mites hiding places near the birds, the alternative housing designs also effectively prevent pesticide treatment of the birds directly. In traditional cages the hens are sprayed from below for northern fowl mite and louse control. That is basically impossible in other housing, where sprays are prevented by structures in the environment such as nest boxes, cage floors, or the ground itself. Here in America we can expect *D. gallinae* to become a serious pest once again as we shift housing away from traditional cages. How bad might it become? It is hard to say, but red mite costs European producers about 130 million euros per year (26, 27) which, as they say, is not chicken feed!

We will be adapting non-traditional housing to control pests based on our understanding of the biology and ecology of those individual species. Especially at a commercial scale we don't know very much about how bad certain pests like red mites may become. Right now we have an urgent need to determine exactly which arthropod pests occur, and exactly where they live, in American alternative housing systems. This probably will provide us with ideas to prevent or suppress parasites culturally. For example, certain types of construction materials inherently are harder to fit precisely (wood, versus metal or plastic) and tend to result in more and better hiding places for off-host parasites. In general, moisture favors parasites too, so we will be trying to minimize that through judicious water use and leak controls. Moisture control always has been critical for flies such as the common house fly (*Musca domestica*). The maggots require moist conditions (above 60% manure moisture). Drier substrates discourage fly oviposition and encourage natural enemy activity that frequently kills nearly all of the flies before they even reach the pest (adult) stage. Aspects like litter material choices and cleanout methods and schedules will need to be tweaked to impede parasites. Examples would include providing parasites insufficient time to complete their life cycles, or keeping poultry houses vacant long enough for the residual parasites to die off before a new flock arrives.

Free-range conditions are a parasite's dream; that kind of condition is where they evolved, after all. Last year the first American study was published on ectoparasite species found on backyard poultry (20). While it did not surprise us, this did surprise most people: the ectoparasite fauna on southern California backyard chickens resembles the diverse fauna on feral chickens in Zimbabwe or Bangladesh. From our observations only two ectoparasite species are at all common on traditionally caged commercial poultry in southern California: the northern fowl mite *O. sylviarum* and the chicken body louse *Menacanthus stramineus*. Backyard chickens had those, plus 5 other

species of lice, lots of sticktight fleas, red mite, and the hideously damaging scaly leg mite. Some of these are potential very nasty pests that would be basically new to our commercial producers. They seldom, if ever, have encountered them since traditional cages were implemented. Unless we are vigilant, some or all of these backyard chicken pests will find their way into the increasingly popular cage-free or especially free range commercial systems. Sticktight fleas, for example, attach to a huge variety of bird and mammal species, including the ubiquitous ground squirrels found in the western USA, and the fleas could very readily invade commercial operations using these alternate hosts. We need to think ahead with regard to surveillance and prevention, and try proactively to generate alternative management strategies for red mites and other parasites likely to occur in alternative housing. The need is especially critical in organic systems, where synthetic pesticides are prohibited.

FUTURE MANAGEMENT OPTIONS

Interested readers can see a pending book chapter (17) for more detail on this topic. Besides cultural controls, some of which are mentioned above, we have a broad range of other choices for control. One interesting cultural possibility for ectoparasites involves beak trimming, which is done to reduce cannibalism, feather picking and feed waste. Hot blade trimming results in permanently blunted beaks, which are inferior tools for grooming. This allows northern fowl mites and lice to reach densities as much as 1-2 orders of magnitude higher than occur on hens that have intact beaks and can groom normally (16). Selection for more docile lines may allow hens to keep their beaks and control their own parasites fairly well, reducing both pest control costs and pesticide exposure. Interestingly, the newer infrared beak trimming methods allow substantial later beak regrowth, including the upper mandible tip overhang that is most useful for killing lice or mites by shearing action (4). Such hens have more parasites than those with a fully intact beak, but still fewer than a hot blade-trimmed bird (21).

Chemical control via pesticides is still common, and approved synthetic pesticides can be used in non-organic settings. Such chemicals tend to be applied to the birds (or delivered in food or water for internal parasites), scattered as toxic baits for flies to eat, or applied as contact pesticides to surfaces such as litter, or to walls and ceilings where pests or parasites may walk or rest. Some newer materials are being used for ectoparasite control on pets and might have promise either on contact or systemically in poultry applications as well, although there is a big difference sometimes between what can be used for a non-food animal (e.g. dogs and cats) versus poultry. Most people involved with pest control think immediately of nerve poisons. Common pesticides like pyrethroids, organophosphates, neonicotinoids and macrocyclic lactones act by interfering with nerve signals. Used properly these materials are safe, but less toxic alternatives exist as well and include growth regulators such as methoprene (e.g. prevent flea egg hatch with even slight surface contact).

With the large amount of European research done on red mites over the past 2 decades, quite a bit has been done on plant essential oils which might be applied to the habitat or perhaps even to the host itself for pest control (5). Many of these are in the category "generally regarded as safe" (GRAS) and would be useable in organic settings and indeed are quite toxic to pests if fresh residues or sprays make contact. They include aromatic materials such as oils of eucalyptus, rosemary, peppermint, garlic, etc. Typically these have a very short activity period (<12-24 h), but some such as thymol may persist and be quite active for up to weeks (12). Importantly, however, they are largely unregulated and also can be expensive, hard to get, and of unknown purity or concentration. They often have not received proper scientific evaluation (e.g. including replications and untreated controls, especially in actual field settings). Good experimental testing is by no means comparable to the testimonials that abound ("wow, this really worked for my chickens"). Regardless, some of these materials are likely to further penetrate the market, and presently are perhaps more attractive for small-scale or organic producers.

Biological control agents kill the parasite or pest by eating it (predators like some beetles and mites attacking fly immatures), parasitizing it (wasps that lay eggs in fly pupae and develop in them, killing the flies before they emerge), or infecting it and causing disease (fungal or bacterial pathogens)(23). These have more potential in a habitat such as manure or litter than they have against parasites that live on or in the host. It is difficult to imagine a 1 cm long predaceous beetle roaming about the feather coat eating lice, and the predaceous beetle larvae need to live someplace too. Still, applying something like a mite pathogen to a host is an attractive possibility. We actually know relatively little about potential arthropod disease agents in natural pest/parasite populations. So there is good potential for exploration (pathogen discovery) and development of existing pathogens for pest control.

Inert dusts such as diatomaceous earth (DE) or kaolin clay kill arthropods by compromising their cuticle. As small as they are, mites or lice have a very high surface:volume ratio and are extremely susceptible to water loss and death by desiccation. The

dusts adhere to the cuticle and absorb surface waxes meant to impede water loss. The damaged cuticle leads to their death. These materials can be delivered in a dust bath containing something like sand as a carrier. Birds dustbathe (24) and a flock with access to concentrated DE can drastically reduce louse or northern fowl mite numbers. Some individual birds don't use the baths and thus their parasites tend to remain, but there is an overall benefit to the flock. Such dustbaths probably need to be recharged with fresh dust about weekly. Dustbathing in the house litter or dirt depressions, even if some DE is clearly seen, is not enough to control the parasites; they need the concentrated dusts in a box (11, 21). The DE is not risk-free, however. The fine particles (less than 10 microns) suspended in air are essentially little pieces of silica (glass) and have real potential to lodge in the lungs of people or animals. Sulfur dust is especially active against mites (11, 22). It is so active that even about 1 g per week per bird, or maybe less, will often eliminate northern fowl mites. The DE is usually fully approved for use in organic settings (inhalation risks aside), but the sulfur may or may not be allowed for organic farms, depending on its source and other inert ingredients. In general organic producers can consult the OMRI (Organic Materials Research Institute) listing for allowed materials.

Vaccines are appealing and have been used and improved for quite some time against coccidia, with potential against other targets (14). Their application for ectoparasites is more problematic, but chickens do develop a certain level of natural immunity against northern fowl mites and perhaps some other blood feeders. As science better understands the nature of immunity, we may be able to develop better and more targeted vaccines, especially against the blood feeders. This idea also applies to the idea of breeding for resistance, although that is probably even further in the future.

Over the past several decades we have tended to lean heavily on a chemical solution to parasites, and perhaps not to think beyond that as much as we should. This also may have to change, as we weave together multiple techniques that individually may be less effective than a pesticide used to be. Integrated pest management (IPM) frequently employs combinations of control methods, rather than using only a single technique, to achieve the desired level of control in the most economical manner. Sometimes an IPM approach, including judicious pesticide use, is both cheaper and more sustainable. In the example used above on beak trimming and mites, for example, the newer infrared trimming method helped, but the combination of modified trimming with DE dustboxes was required to get the numbers of northern fowl mites below what is thought to be an appropriate damage threshold of 100 mites per hen (18, 21).

Finally, we must never forget the basic idea that biosecurity is vital. This idea is known well by poultrymen with regard to avian pathogens, but the best defense against parasites similarly is never getting them in the first place. The same procedures and precautions meant to keep out disease agents, or keep them from spreading on a farm, apply to parasites.

THE IMPORTANCE OF MONITORING AND IPM

Like other farmers, poultry producers are businessmen and have a lot to do. It requires little thought to implement controls such as antiparasitic pesticide treatments on a regular schedule. Maybe this actually works in some cases, but it can be a big waste of resources if the pest is not there, or is not yet found in damaging numbers. Most of the time a small number of parasites does not cause enough damage to be worth treating, in the sense that the treatment will at least pay for itself down the line in improved performance (better egg production, etc. than would occur if the birds were untreated). Pesticide treatment alone often does not completely eliminate the pest either. Certain parasites may not be worth treating at all; some species of lice merely eat some of the feather material but tend not to reach high populations, especially if the hosts can groom well. If poultry producers are concerned with eliminating any possibility of animal discomfort from parasites, regardless of profit, that is its own case. Given recent trends it would not be surprising, actually, for welfare-based legislation at some point in the future to require pest control solely for animal comfort. Presently, however, the IPM concept usually is linked to the idea that one must control a pest when it will be profitable to do so. Management is initiated when parasite abundance exceeds a level where the financial damage from parasites is expected to be greater than the cost of parasite control (pesticide, labor, equipment). That treatment point is called the economic threshold.

For most parasites we still lack the scientific database to generate such thresholds, or even practical monitoring methods. We do have such methods for certain environmental pests such as house flies (6) or litter beetles (25). Northern fowl mites provide an example of how it might work for a parasite (18). These mites do best in cool weather and are worst in new (immunologically naïve) flocks under the stress of reaching peak egg production. They can be monitored by randomly checking the vent feathers of hens, estimating proportions of hens infested (presence-absence sampling)(7). Checking as few as 10 well-distributed birds in a house may generate a

treatment (all are infested at some level) or a no-treatment decision (none are infested). A less exact method, but much better than no monitoring, might be to check 100 eggs in the roll outs scattered throughout the house, treating when 4/100 have any visible mites (Mullens, unpublished). Treating when mite infestations are light (25% prevalence and fewer than 10 mites per hen average, or about 4/100 eggs with mites) can result in excellent control, while treating once mites are heavy may result in rather poor control. So timing matters and it is economically worth regular (weekly) sampling for mites, especially when one expects trouble (young flock in cool weather for a farm with a mite history). Strategic northern fowl mite control efforts also should mitigate or prevent worker complaints from mites in the houses (as human pests), which can be a real concern as well. Perhaps once mites have declined due to immune responses or hot weather, the potential for economic damage is less and vigilance might be relaxed. On the other hand, on a farm with excellent biosecurity and no mite history, occasional checks early in a flock's life may suffice.

CONCLUSION

We live in a time of great change in animal agriculture, including new challenges for pest management. The important thing is for parasite control to be part of the discussion as we adopt new housing or management styles. Producers can and will adapt to these changes, but they need scientists to help them see the issues coming and to try to be proactive in developing solutions.

REFERENCES

1. Axtell, R. C. and J. J. Arends. Ecology and management of arthropod pests of poultry. Ann. Rev. Entomol. 35: 101-126. 1990.

2. Bishopp, F. C. and H. P. Wood. Mites and lice on poultry. USDA Farmer's Bull. 801. 19 pp. 1931.

3. Blatchford, R. A., R. M. Fulton and J. A. Mench. The utilization of the welfare quality® assessment for determining laying hen condition across three housing systems. Poultry Sci. 95: 154-163. 2016.

4. Clayton, D. H., B. R. Moyer, S. E. Bush, T. G. Jones, D. W. Gardiner, B. B. Rhodes, and F. Goller. Adaptive significance of avian beak morphology for ectoparasite control. Proc. Biol. Sci. 272:811–817. 2005.

5. George, D. R., Finn, R. D., Graham, K. M., Sparagano, O. A. E., and Olivier, A. E. Present and future potential of plant-derived products to control arthropods of veterinary and medical significance. Par. Vect. 7: doi: 10.1186/1756-3305-7-28. 2014.

6. Gerry, A. C., G. E. Higginbotham, L. N. Periera, A. Lam and C. R. Shelton. Evaluation of surveillance methods for monitoring house fly abundance and activity on large commercial dairy operations. J. Econ. Entomol. 104: 1093-1102. 2011.

7. Harris, M. A., M. J. Brewer and J. A. Meyer. Presence-absence sampling plan for northern fowl mite, *Ornithonyssus sylviarum* (Acari: Macronyssidae), on caged-layer hens. J. Econ. Entomol. 93: 544-549. 2000.

8. Hoglund, J., H. Nordenfors, and A. Uggla. Prevalence of the poultry red mite, *Dermanyssus gallinae*, in different types of production systems for egg layers in Sweden. Poult. Sci. 74: 1793-1798. 1995.

9. Lay, D. C., M. Fulton, P. Y. Hester, D. M. Karcher, J. Kjaer, J. A. Mench, B. A. Mullens, R. C. Newberry, C. J. Nicol, N. P. O'Sullivan, and R. E. Porter. Hen welfare in different housing systems. Poult. Sci. 90: 278-294. 2011.

10. Malone, T. and J. L. Lusk. Putting the chicken before the egg price: an ex post analysis of California's battery cage ban. J. Agric. Res. Econ. 41: 518-532. 2016.

11. Martin, C. D. and B. A. Mullens. Housing and dustbathing effects on northern fowl mites (*Ornithonyssus sylviarum*) and chicken body lice (*Menacanthus stramineus*) on hens. Med. Vet. Entomol. 26: 323-333. 2012.

12. Massoumi, F., M. R. Youssefi, and M. A. Tabari. Combination of carvacrol and thymol against the poultry red mite (*Dermanyssus gallinae*). Parasitol. Res. 115: 4239-4243. 2016.

13. McDougald, L. R. Protozoal infections. IN Swain, D. E. (ed.). Diseases of Poultry. 13th ed. Blackwell Publ. Co., Ames. Iowa. 2013.

14. Meunier, M., M. Chemaly and D. Dory. DNA vaccination of poultry: the current status in 2015. Vaccine 34: 202-211. 2016.

15. Mul, M. F., T. Van Niejirk, J. Chirico, V. Maurer, O. Kilpinen, O. Sparagano, B. Thind, J. Zoons, D. Moore, B. Bell, A. G. Gjevre and C. Chave. Control methods for *Dermanyssus gallinae* in systems for laying hens: results of an international seminar. World's Poultry J. 65: 589-599. 2009.

16. Mullens, B. A., B. L. Chen and J. P. Owen. Beak condition and cage density determine abundance and spatial distribution of northern fowl mites, *Ornithonyssus sylviarum*, and chicken body lice, *Menacanthus stramineus*, on caged laying hens. Poultry Sci. 89: 2565-2572. 2010.

17. Mullens, B. A. and A. C. Murillo. The Future of Poultry Pest Management. IN Mench, J. A. (ed.). Advances in Poultry Welfare. Elsevier. (in press).

18. Mullens, B. A., J. P. Owen, D. R. Kuney, C. E. Szijj and K. A. Klingler. Temporal changes in distribution, prevalence and intensity of northern fowl

mite (*Ornithonyssus sylviarum*) parasitism in commercial caged laying hens, with a comprehensive economic analysis of parasite impact. Vet. Parasitol. 160: 116-133. 2009.

19. Mullens, B. A., R. K. Velten, N. C. Hinkle, D. R. Kuney and C. E. Szijj. Acaricide resistance in northern fowl mite (*Ornithonyssus sylviarum*) populations on caged layer operations in southern California. Poultry Sci. 83: 365-374. 2004.

20. Murillo, A. C and B. A. Mullens. Diversity and prevalence of ectoparasites on backyard chicken flocks in California. J. Med. Entomol. 53: 707-711. 2016a.

21. Murillo, A. C. and B. A. Mullens. Timing diatomaceous earth-filled dustbox use for management of northern fowl mites (Acari: Macronyssidae) in cage-free poultry systems. J. Econ. Entomol. 109: 2572-2579. 2016b.

22. Murillo, A. C. and B. A. Mullens. Sulfur dust bag: a novel technique for ectoparasite control in poultry systems. J. Econ. Entomol. 109: 2229-2233. 2016c.

23. Oliveira, D. G. P., L. F. A. Alves and D. R. Gomez. Advances and perspectives of the use of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* for the control of arthropod pests in poulry production. Braz. J. Poultry Sci. 16: 1-12. 2015.

24. Olsson, I. A. S. and L. J. Keeling. Why in earth? Dustbathing behaviour in jungle and domestic fowl reviewed from a Tinbergian and animal welfare perspective. Appl. Anim. Behav. Sci. 93: 259-282. 2005.

25. Safrit, R. D. and R. C. Axtell. Evaluations of sampling methods for darkling beetles (*Alphitobius diaperinus*) in the litter of turkey and broiler houses. Poult. Sci. 63: 2368-2375. 1984.

26. Sparagano, O. A. E., D. R. George, D. W. J. Harrington, and A. Giangaspero. Significance and control of the poultry red mite, *Dermanyssus gallinae*. Annu. Rev. Entomol. 59: 447–466. 2014.

27. Sparagano, O. A. Pavlecevic, T. Murano, A. Camarda, H. Sahibi, O. Kilpinen, M. Mul, R. van Emous, S. le Bouquin, K. Hoel and M. A. Cafiero. Prevalence and key figures for the poultry red mite *Dermanyssus gallinae* infections in poultry farm systems. Exp. Appl. Acarol. 48: 3-10. 2009.

28. Vaarst, M., S. Steenfeldt and K. Horsted. Sustainable development perspectives on poultry production. World's Poult. Sci. J. 71: 609-620. 2015.

29. Yazwinski, T. and C. Tucker. Nematodes and acanthocephalans. IN Y. Saif. (ed.). Diseases of Poultry. 12th ed., Blackwell Publ., Ames, Iowa. 2008.

MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE OF *CAMPYLOBACTER* SPECIES ISOLATED FROM POULTRY

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ABSTRACT

Campylobacter species are among the leading causes of foodborne diseases in humans worldwide. Poultry and poultry products are the major source of *Campylobacter* infection in humans. The increasing trend of antimicrobial resistance in bacterial pathogens, including *Campylobacter*, within the food chain has become a serious public health concern. The purpose of this study was to analyze the genetic diversity and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from egg layers. A total of 35 *Campylobacter* isolates i.e. *C. jejuni* (n=11) and *C. coli* (n=24) were selected from six different layer farms. Antimicrobial susceptibility was performed by minimum inhibition concentration (MIC). Multi-locus Sequence Typing (MLST) was performed to find the genetic diversity among the *Campylobacter* species isolated from layers. Of the *C. coli* isolates selected for genetic analysis, 87.5% (21/24) were resistant to either erythromycin or spectinomycin and 12.5% (3/24) were highly resistant to chlortetracycline. Among *C. jejuni* isolates, 72.7% (8/11) were resistant to erythromycin or ciprofloxacin while 27.3% (3/11) were highly resistant to chlortetracycline. Eleven different sequence types (STs) were identified, including seven STs for *C. jejuni* and four for *C. coli*. The most common *C. jejuni* STs were ST-443 and ST-353 while ST-828 was the predominated ST for *C. coli*. Age-wise distribution for *C. jejuni* revealed that ST- 51 was the dominant sequence type followed by ST-50 while in *C. coli,* ST-829 was prevalent sequence type followed by ST-825 in all age groups of layers. No significant association was observed between sequence types and different farming systems. *Campylobacter* species are circulating in layer birds and the most common clonal complex in layers is ST-828 for *C. coli* and ST-443 and ST-353 for *C. jejuni*.

Thermo-tolerant *Campylobacter* is one of the most well-known causes of food borne diseases in humans worldwide. The digestive tract of all warm blooded animals is a significant reservoir for these bacteria resulting in huge economic losses to any country because of restrictions in international trade of meat and meat products. Thermo-tolerant *Campylobacter* is one of the most well-known causes of food borne diseases in humans worldwide. The digestive tract of all warm blooded animals is a significant reservoir for these bacteria resulting in huge economic losses to any country because of restrictions in international trade of meat and meat products.

INTRODUCTION

Thermo-tolerant *Campylobacters* are one of the leading bacterial causes of foodborne gastrointestinal diseases. An estimate of nine and 0.2 million cases occurs in United States (1) and European Union every year, respectively. *Campylobacters* have zoonotic potential and many cases have been reported from developed as well as in under developed countries. These bacteria are part of the intestinal flora in most warm-blooded animals (2, 3). The organisms have frequently been isolated from meat and meat products which are the major sources of human infection. The pathogen survives in under-cooked meat, meat products, raw milk, and dairy products (4, 5).

Though *Campylobacter* is frequently found in fresh water and in food animals, such as cows and in wild animals, the role that these sources play in the epidemiology of Campylobacteriosis, or how transmission to human hosts would commonly occur is not yet clear (6, 7).

Normally, *Campylobacter* survives at a high temperature of 42˚C and a microaerophilic environment for growth. Conventional microbiological methods for isolation of this bacteria

are laborious, time consuming, and not reliable (8). Inter and intra species diversity within the *Campylobacter* genus makes identification difficult at the species level (9-12). A number of molecular techniques have been reported for rapid identification of *Campylobacter* (9, 13). A new generation of molecular typing methodologies will help to further characterize sources of *Campylobacter* infection. Molecular typing methodologies have been instrumental in enhancing epidemiological investigations. These techniques enhance our understandings on sources of sporadic infections with *Campylobacter* spp. by providing information on the genetic subtypes in circulation. Advanced molecular techniques such as pulsed-field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) have been used to differentiate the species and linked their transmission (14). It is used to develop strategies to mitigate incidence of *Campylobacter* in food chain (15). MLST has found to be effective method for genotyping of *Campylobacter,* where seven housekeeping genes are amplified by PCR (16). The technique has high power of discrimination by comparing available DNA sequences for genotyping (17). It made it possible to compare *Campylobacter* isolated from different animal species human cases sequence types (STs) and clonal complexes (CCs) (5). Recent advances have overcome its constraints of being time consuming, expensive and laborious and is now affordable and time efficient (12). Further advantages include high reproducibility, easy to interpret results (18), and a large database of isolates form around the world available on a PubMLST database.

This study was designed to conduct molecular subtyping and to investigate antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolates form egg layer birds.

MATERIALS AND METHODS

Bacterial isolates. A total of 360 fresh fecal samples were collected from six farms (Organic and conventional farms) in north western Ohio, during November 2014 to January 2015. Three farms from each farming system were selected and a total of 60 fecal samples were collected from each farm. Briefly, one gram of fecal samples were suspended in 9 mL of Preston broth (SR0117, Oxoid) with antibiotics (SR0232, Oxoid). The tubes were incubated under microaerophilic conditions at 37°C for four hours followed by 42°C for 44 hours. After 48 hours of incubation the enriched broth was sub-cultured on mCCDA (modified charcoal cefoperazone deoxycholate agar) supplemented with antibiotics (SR0155, Oxoid) and incubated for another 48 hours under microaerophilic conditions. *Campylobacter* like colonies were further purified on Muller-Hintion agar with 7% defibrinated sheep blood (19).

DNA extraction. DNA was extracted by QIAamp DNA Mini Kit (cat# 51306 Qiagen, USA) according to manufacturer's instructions. The extracted DNA was stored at -20°C till further use.

PCR amplification. Amplification was carried out in 25µL reaction. The reaction consists of 12.5µL PCR mastermix (Lucigen cat # 33311, USA), 1µL of forward and 1µL of reverse primers for all the three genes (*16SrRNA*, *mapA* and *ceuE*) and 4.5µL of nucleases free water. The tubes were placed in T100 thermal cycler (BioRad USA) with protocol of initial denaturation at 95˚C for 10 min, denaturation for one min at 94˚C, annealing at 48˚C for one min, extension for one min at 72˚C, final extension at 72˚C for 10 min and 4˚C for infinite time. The machine was set for 35 cycles to amplify the DNA of interest. PCR product were visualized by gel electrophoresis under UV light in 1.2 % agarose gel.

Antimicrobial susceptibility. Antimicrobial susceptibility was performed using the broth microdilution method. All the isolates were tested against five antibiotics i.e., ciprofloxacin, erythromycin, spectinomycin, tetracycline and tylosine. *C. jejuni* (81–176) and *C.coli* (ATCC 33559) were used as control strains in this experiment. Minimum inhibitory concentrations and resistance breakpoints were determined as described previously (20). Isolates that were resistant to three or more antimicrobial classes were considered as multidrug resistant (MDR) (21).

Detection of antibiotic resistance genes. Multiplex-PCR was used to for detection of four antimicrobial resistance genes: aph-3-1 (aminoglycoside resistance), blaOXA-61 (ampicillin resistance), cmeB (encoding the multidrug efflux pump) and tet(O) (tetracycline resistance) in the *Campylobacter* isolates (22).

Multi locus sequence typing. A total of 35 *Campylobacter* isolates (*C. jejuni*=11 and *C.coli*= 24) were selected for characterization by multi locus sequence typing (MLST). The isolates with profile of resistance to antimicrobial agents were selected for this study. MLST was initiated by selecting primers for *C. jejuni* and *C.coli* from PUBMLST website (http://pubmlst.org/*Campylobacter*). The primers optimized for seven housekeeping genes *i.e., aspA* (aspartaseA), *glnA* (glutamine synthase), *gltA* (citratesynthase), *glyA* (serinehydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase), and *uncA* (ATP synthase alpha subunit) (17). Amplification was carried out in 25µL reaction mixture: 12.5µL PCR mastermix (Lucigen cat#33311), 1µL of both forward

and reverse primers and 8.5µL of nucleases free water. The amplification was carried out in T100 Thermal Cycler (BioRad USA) with protocol of initial denaturation at 95˚C for 10 minutes, denaturation at 94˚C for two minutes, annealing at 50˚C (*C.coli*) 49˚C (*C. jejuni*) for one minute, extension at 72˚C for one minute, final extension at 72˚C for 10 minutes and hold at 4˚C for unlimited time. Distilled water was included as negative control. The PCR products were visualized using 1.2% (w/v) agarose gel electrophoresis. The PCR products were cleaned up using the Qiagen PCR product purification kit (Qiagen USA), and Sanger sequencing of genes was conducted by University of Minnesota Genomic Centre (UMGC) Minnesota USA. The sequences were assembled using the assemblage implemented in Sequencher 5.4 software. All allelic sequences were queried against the *C. jejuni* and *C.coli* MLST database. Alleles already present in the database were assigned the numbers given there; novel alleles and sequence types (STs) were submitted to the *C. jejuni* MLST database and assigned new numbers. Sequence types were assigned into genetically related clusters called clonal complexes (CCs), based on sharing four or more alleles with the central genotype.

RESULTS

Out of 360 fecal samples collected, 248 samples were confirmed for *Campylobacter* positive from both farming systems. Equal number (124) of *Campylobacter* were isolated from each production system. High prevalence of *C.coli* was recorded as compared to *C. jejuni*. Highest resistance was seen in *Campylobacter* isolates against tetracycline in both systems while lowest resistance was against ciprofloxacin. A total of 45 isolates were found as multi drug resistant. Eighteen were from conventional farming and 27 were from organic farming. In overall *Campylobacter* isolates, *tet(O)* and *cmeB* are frequently detected than *aph-3-1* and *blaOX-61* (23).

In *C. coli* isolates selected for genetic analysis, 87.5% (21/24) were resistant to either erythromycin or spectinomycin and 12.5% (3/24) were highly resistant to chlortetracycline. Among *C. jejuni* isolates, 72.7% (8/11) were resistant to erythromycin or ciprofloxacin while 27.3% (3/11) were highly resistant to chlortetracycline. Eleven different sequences types (STs) were identified, including 7 STs for *C. jejuni* and 4 for *C. coli*. The most common clonal complex (CCs) in *C. jejuni* were the ST-443 and ST-353 while in *C.coli* ST-828 was the predominated clonal complex. Age-wise distribution for *C. jejuni* revealed that ST-51 was the dominant sequence type followed by ST-50 while in *C.coli,* ST-829 was prevalent sequence type followed by ST-825 in all age groups of layers. No significant association was observed between sequence types and different farming systems. *Campylobacter* species are circulating in layer birds and the most common clonal complex in layer ST-828 for *C coli* and ST-443, ST-353 for *C. jejuni*.

REFERENCES

1. Painter, J.A. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbreak Data, United States, 1998–2008-Volume 19, Number 3—March 2013-Emerging Infectious Disease journal-CDC. 2013.

2. Bae, W., D.D. Hancock, D.R. Call, Y.H. Park, A.C. Berge, R.M. Finger, W.M. Sischo, and T.E. Besser Dissemination of antimicrobial resistant strains of *Campylobacter coli* and *Campylobacter jejuni* among cattle in Washington State and California. Veterinary microbiology 122:306-315. 2007.

3. Englen, M.D., A.E. Hill, D.A. Dargatz, S.R. Ladely, and P.J. Fedorka-Cray Prevalence and antimicrobial resistance of *Campylobacter* in US dairy cattle. Journal of applied microbiology 102:1570-1577. 2007.

4. Frederick, A., and N. Huda Canipylobacter in Poultry: Incidences and Possible Control Measures. 2011.

5. Humphrey, T., S. O'Brien, and M. Madsen *Campylobacter*s as zoonotic pathogens: a food production perspective. International journal of food microbiology 117:237-257. 2007.

6. Taylor, E.V., K.M. Herman, E.C. Ailes, C. Fitzgerald, J.S. Yoder, B.E. Mahon, and R.V. Tauxe Common source outbreaks of *Campylobacter* infection in the USA, 1997-2008. Epidemiology and infection 141:987-996. 2013.

7. Friesema, I., R. De Boer, E. Duizer, L. Kortbeek, D. Notermans, A. Smeulders, J. Bogerman, M. Pronk, J. Uil, and K. Brinkman Aetiology of acute gastroenteritis in adults requiring hospitalization in The Netherlands. Epidemiology and infection 140:1780-1786. 2012.

8. On, S.L. Taxonomy of *Campylobacter*, Arcobacter, Helicobacter and related bacteria: current status, future prospects and immediate concerns. Symp Ser Soc Appl Microbiol:1S-15S. 2001.

9. Denis, M., C. Soumet, K. Rivoal, G. Ermel, D. Blivet, G. Salvat, and P. *Coli*n Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C.coli*. Letters in applied microbiology 29:406-410. 1999.

10. On, S.L., and P.J. Jordan Evaluation of 11 PCR assays for species-level identification of *Campylobacter jejuni* and *Campylobacter coli*. Journal of clinical microbiology 41:330-336. 2003.

11. Dedieu, L., J.M. Pages, and J.M. Bolla Use of the omp50 gene for identification of *Campylobacter* species by PCR. Journal of clinical microbiology 42:2301-2305. 2004.

12. Sheppard, S.K., K.A. Jolley, and M.C. Maiden A Gene-By-Gene Approach to Bacterial Population Genomics: Whole Genome MLST of *Campylobacter*. Genes 3:261-277. 2012.

13. Denis, M., J. Refregier-Petton, M.J. Laisney, G. Ermel, and G. Salvat *Campylobacter* contamination in French chicken production from farm to consumers. Use of a PCR assay for detection and identification of *Campylobacter jejuni* and Camp. *coli*. Journal of applied microbiology 91:255-267. 2001.

14. Levesque, S., E. Frost, R.D. Arbeit, and S. Michaud Multilocus sequence typing of Campylobacter *jejuni* isolates from humans, chickens, raw milk, and environmental water in Quebec, Canada. Journal of clinical microbiology 46:3404-3411. 2008.

15. Moffatt, C.R., S. Cameron, L. Mickan, and R.C. Givney *Campylobacter jejuni* gastroenteritis at an Australian boarding school: consistency between epidemiology, flaA typing, and multilocus sequence typing. Foodborne pathogens and disease 7:1285-1290. 2010.

16. Colles, F.M., K. Jones, R.M. Harding, and M.C. Maiden Genetic diversity of *Campylobacter jejuni* isolates from farm animals and the farm environment. Applied and environmental microbiology 69:7409-7413. 2003.

17. Dingle, K.E., F.M. Colles, D.R. Wareing, R. Ure, A.J. Fox, F.E. Bolton, H.J. Bootsma, R.J. Willems, R. Urwin, and M.C. Maiden Multilocus sequence typing system for *Campylobacter jejuni*. Journal of clinical microbiology 39:14-23. 2001.

18. Levesque, S., S. Michaud, R.D. Arbeit, and E.H. Frost High-resolution melting system to perform multilocus sequence typing of *Campylobacter jejuni*. PloS one 6:e16167. 2011.

19. Kashoma, I.P., Kassem, II, J. John, B.M. Kessy, W. Gebreyes, R.R. Kazwala, and G. Rajashekara Prevalence and Antimicrobial Resistance of *Campylobacter* Isolated from Dressed Beef Carcasses and Raw Milk in Tanzania. Microb Drug Resist 22:40-52. 2016.

20. Ge, B., F. Wang, M. Sjölund-Karlsson, and P.F. McDermott Antimicrobial resistance in *Campylobacter*: susceptibility testing methods and resistance trends. Journal of microbiological methods 95:57-67. 2013.

21. Hakanen, A.J., M. Lehtopolku, A. Siitonen, P. Huovinen, and P. Kotilainen Multidrug resistance in *Campylobacter jejuni* strains collected from Finnish patients during 1995–2000. Journal of Antimicrobial Chemotherapy 52:1035-1039. 2003.

22. Obeng, A., H. Rickard, M. Sexton, Y. Pang, H. Peng, and M. Barton Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. Journal of applied microbiology 113:294-307. 2012.

23. Kassem, II, O. Kehinde, A. Kumar, and G. Rajashekara Antimicrobial-Resistant *Campylobacter* in Organically and Conventionally Raised Layer Chickens. Foodborne pathogens and disease 14:29-34. 2017.

Table 1. Distribution of *C. jejuni* and *C.coli* sequence types (STs), clonal complexes (CCs).

ID	Specie	System	*aspA*	*glnA*	*gltA*	*glyA*	*tkt*	*pgm*	*uncA*	STs	CCs
NC001	*C. jejuni*	CF	2	1	12	3	1	2	5	50	ST-21 complex
NC002	*C. jejuni*	CF	4	7	40	4	51	42	1	267	ST-283complex
NC003	*C. jejuni*	CF	7	17	2	15	3	23	12	51	ST-443 complex
NC004	*C. jejuni*	CF	7	17	2	15	3	23	12	51	ST-443 complex
NC005	*C. jejuni*	CF	2	1	12	3	1	2	5	50	ST-21 complex
NC006	*C. jejuni*	CF	7	17	5	2	3	10	6	353	ST-353 complex
NC007	*C. jejuni*	CF	7	17	2	15	3	23	12	51	ST-443 complex
NC008	*C. jejuni*	OG	9	2	4	62	5	4	17	929	ST-257 complex
NC009	*C. jejuni*	CF	2	1	12	15	1	2	5	5252	ST-21 complex
NC010	*C. jejuni*	CF	7	17	2	3	3	23	12	7234	ST-443 complex
NC011	*C. jejuni*	CF	7	112	5	2	67	38	6	2838	ST-353 complex
										2934	ST-353 complex
										7729	ST-353 complex
NC013	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC014	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC015	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC016	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC017	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC018	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC019	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC020	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC021	*C.coli*	CF	103	110	337	140	164	56	120	1239	
										2633	ST-1150 complex
										2637	ST-1150 complex
										7265	
NC022	*C.coli*	CF	33	39	30	82	43	113	17	829	ST-828 complex
NC023	*C.coli*	CF	33	39	30	82	47	113	17	825	ST-828 complex
NC024	*C.coli*	CF	33	39	30	82	47	113	17	825	ST-828 complex
NC025	*C.coli*	CF	33	39	30	82	47	113	17	825	ST-828 complex
NC026	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC027	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC028	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC029	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC030	*C.coli*	OR	32	38	30	82	43	152	17	900	
										1624	ST-828 complex
										2878	ST-828 complex
										6162	ST-828 complex
										6797	ST-828 complex
										7681	
NC031	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC032	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC033	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC034	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC035	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex
NC036	*C.coli*	OR	33	39	30	82	43	113	17	829	ST-828 complex

CF= Conventional farming system, **OG**=Organic farming system

DNA SEQUENCING CONFIRMATION OF *MYCOPLASMA GALLISEPTICUM* FROM MAGELLANIC PENGUINS (*SPHENISCUS MAGELLANICUS*)

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SUMMARY

Mycoplasma isolates from penguins (*Spheniscus magellanicus*) rescued on shores of Rio de Janeiro city, RJ state, Brazil, and PCR positive to MG were now confirmed by sequencing with 99% significant alignments to others MG sequences in Gene Bank. The 10 isolate samples yielded the same sequence among themselves with 100% of identity, which imply a common source of infection. The blast analysis exhibited strong relationship with *M. gallisepticum* sequences in GenBank database, but the first hit was to an untyped sequence (GenBank, *Mycoplasma* spp. 56A97 – LN811535) while the twentieth hit to *M. imitans*. How the investigated penguins got infected and weather our isolate is indeed *M. gallisepticum* and/or pathogenic or transmissible to poultry, still need to be clarified.

INTRODUCTION

Mycoplasmas have been isolated from several species of birds, including domestic and wild ones (1, 3, 11). In aquatic birds, there have been reports of isolation of mycoplasmas in ducks (7), swans, geese, night heron (12). Mycoplasma species are important because they can cause respiratory, articular and reproductive diseases, besides creating a favorable environment for the development of secondary infection (5, 8). In penguins, there have been also reports on mycoplasma isolation (1, 4, 2, 11, 14), including *Mycoplasma gallisepticum,* based on specific PCR with two different pairs of primers (9, 15) in Magellanic penguin (*Spheniscus magellanicus*) rescued in Rio de Janeiro beaches (2), besides a report on mycoplasma resembling *M. gallisepticum* (14). Because *M. gallisepticum* is an undoubtedly pathogenic to poultry (7, 8), DNA sequencing was done in 10 penguin isolates diagnosed by PCR and kept in our laboratory.

MATERIALS AND METHODS

A total of 10 *M. gallisepticum* PCR typed isolates from Magellanic penguins (Spheniscus magellanicus), stored at -20ºC were processed for DNA extraction by applying DNeasy Blood & Tissue Kit (Qiagen) for the purpose of sequencing by the Sanger method. DNA amplification was performed by primers 593 - 5' GTTTGATCCTGGCTCAGGAYDAACG 3'and 620B - 5'GAAAGGAGGTRWTCCACYCSCAC3' that target the 16S ribossomal gene, comprising 1460 bp amplicon (10).

These PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and quantified by Qubit dsDNA HS Assay Kit (Applied Biosystems). Each sequencing reaction (20ng of PCR product) was carried out by cycle sequencing with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to manufacturer's instructions and analyzed in an Applied Biosystems ABI Prism 3730 automated DNA sequencer.

To each sample, six sequencing reactions were performed, with those PCR primers, plus internal primers 388 5'CCARACTCCTACGGRAGGCAGC3' and 390B 5´CTTGTGCGGGYYCCCGTCAATTC3' and their reverse complement sequences, i.e., 388-RC (5' GCTGCCTYCCGTAGGAGTYTGG 3') 390B-RC (5'GAATTGACGGGRRCCCGCACAAG 3').

Sequences were assembled and edited with SeqMan, DNAStar software 7.0 version (Lasergene) and the contig sequences were blasted with the GenBank database (http://www.ncbi.nlm.nih.gov/blast).

RESULTS AND DISCUSSIONS

The 10 isolate samples yielded the same sequence among themselves with 100% of identity, which suggests a common source of infection. The first reports regarding isolation of mycoplasma from penguins had found no similarities with *M. gallisepticum* (1, 4, 11). The blast analysis, of our 10 isolates consensus sequence, exhibited strong

relationship with *M. gallisepticum* sequences, but the first hit was to an untyped sequence (GenBank, *Mycoplasma* sp. 56A97 – LN811535) and the twentieth hit to *M. imitans* (Table 1). As, all sequences exhibited 99% identity, the hit order took account to the maximum score, that is, the numerical value that describes the overall quality of the alignment.

The comparison of our penguin isolate consensus with 56A97 – LN811535, in SeqMan DNAStar software, showed just one difference marked by a transition of cytosine to thymine in nucleotide 990 of the 1460bp alignment, extended by 593 and 620B primers. Comparing the same isolate consensus with the second blast hit, *M. gallisepticum* S6 - CP006916 we found two differences (nucleotides 140 and 587) while to the twentieth hit, *M. imitans*, three differences were seen (nucleotides 166, 420 and 437). Yavari and her colleagues strongly suggested that 56A97 isolate was distinct from *M. gallisepticum* and *M. imitans* (15).

CONCLUSIONS

Based on our findings and information from the literature, we are not sure that our mycoplasma penguin isolate belongs to *M. gallisepticum* species. More studies are needed to accomplish the real taxonomic status of this strain.

REFERENCES

1. Banks JC, Cary SC, Hogg ID. The phylogeography of adelie penguin faecal flora. *Environmental Microbiology*, v.11, n.3, p.577-588. 2009.

2. Barreto ML, et al. *Mycoplasma Gallisepticum* by PCR in Glucose Fermenting Mycoplasma Isolates from Magellanic Penguins (Spheniscus Magellanicus) in Brazil. In: 62nd Western Poultry Disease Conference, Sacramento, CA, USA. Proceedings of the 62nd WPDC, p. 73. March 25-27, 2013 73p. Sacramento, CA, USA. 2013.

3. Forsyth MH, et al. *Mycoplasma sturni* sp. nov., from the conjunctiva of a European starling (*Sturnus vulgaris*). *International Journal of Systematic Bacteriology*, v.46, n.3, p.716–719.

4. Frasca SJr, et al. 2005. Isolation and characterization of *Mycoplasma sphenisci* sp. nov. from the choana of an Aquarium-Reared Jackass Penguin (*Spheniscus demersus*). *Journal of Clinical Microbiology*, v.43, n.6, p.2976–2979. 1996.

5. Friend M, Franson JC. Mycoplasmosis, p.115-119. In Field manual of wildlife diseases: general field procedures and diseases of birds. Biological Resources Division, Geological Survey (U.S.): Washington, DC. 1999.

6. Goldberg DR, et al. The occurrence of mycoplasmas in selected wild north American waterflow. *Journal of Wildlife Diseaes*, v.31, n.3, p.364-371. 1995.

7. Nascimento ER, Pereira VLA. Micoplasmoses, p. 485-495. In Berchieri Jr. A., Silva EN, Fábio JD, Sesti L, Zuanaze MAF (ed), Doenças das Aves, 2nd ed. FACTA (Fundação APINCO de Ciência e Tecnologia Avícolas), Campinas, SP. 2009.

8. Nascimento ER, et al. Avian mycoplasmosis update. *Revista Brasileira de Ciência Avícola*, v.7, n.1, p.1-9. 2005.

9. Nascimento ER, et al. Aprimoramento da PCR para *Mycoplasma gallisepticum* pelo encurtamento do "amplicon" e ajustes no processamento da amostra. *Acta Scientiae Veterinariae*, Porto Alegre, v.33, n.3, p.297-301. 2005.

10. Petersson B, M, Uhlen MM, Johansson K-E. Phylogeny of Some Mycoplasmas from Ruminants Based on 16s rRNA Sequences and Definition of a New Cluster within the Hominis Group. International Journal of Systematic Bacteriology, v.46, n.4, p. 1093-1098. 1996.

11. Poveda JB, et al. Isolations of mycoplasmas from a buzzard, falcons and vultures. *Avian Pathology*, v.19, n.4, p.779-783. 1990.

12. Shimizu T, Numano K, Uchida K. Isolation and identification of the mycoplasma from various birds: an ecological study. *Japanese Journal of Veterinary Science*, v.41, n.3, p.273-282. 1979.

13. Souza LDC, et al. 2012. *Mycoplasma sphenisci* in Magellanic Penguins (*Spheniscus magellanicus*) found on beaches of Rio de Janeiro State, Brazil. In: 19th Congress of International Organization of Mycoplasmology, Toulouse, FR. IOM 2012 Programme & Abstracts, p.117. 2012.

14. Yavari, C.A., Studies on a Mycoplasma gallisepticum-like organism isolated from the Humboldt penguin (Spheniscus humboldti), (unpublished PhD thesis, University of Liverpool). 2010.

15. World Organisation for Animal Health - OIE. Avian Mycoplasmosis (*Mycoplasma gallisepticum, M. synoviae*). In: OIE Terrestrial Manual, Chapter 2.3.5, p.482-496, 2008.

Table 1. Twenty major scores of blast analysis from mycoplasma penguin isolate consensus in Genbank database.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycoplasma sp. 56A97 DNA containing 16S rRNA gene (partial) and 16S-23S intergenic spacer region, strain 56A97	2691	2691	100%	0	99%	LN811535.1
Mycoplasma gallisepticum S6, complete genome	2686	5372	100%	0	99%	CP006916.3
Mycoplasma gallisepticum CA06_2006.052-5-2P, complete genome	2686	5366	100%	0	99%	CP003512.1
Mycoplasma gallisepticum WI01_2001.043-13-2P, complete genome	2686	5366	100%	0	99%	CP003510.1
Mycoplasma gallisepticum NY01_2001.047-5-1P, complete genome	2686	5361	100%	0	99%	CP003509.1
Mycoplasma gallisepticum NC96_1596-4-2P, complete genome	2686	5366	100%	0	99%	CP003508.1
Mycoplasma gallisepticum NC95_13295-2-2P, complete genome	2686	5366	100%	0	99%	CP003507.1
Mycoplasma gallisepticum VA94_7994-1-7P, complete genome	2686	5366	100%	0	99%	CP003506.1
Mycoplasma gallisepticum NC08_2008.031-4-3P, complete genome	2680	5361	100%	0	99%	CP003513.1
Mycoplasma gallisepticum NC06_2006.080-5-2P, complete genome	2680	5361	100%	0	99%	CP003511.1
Mycoplasma gallisepticum str. F, complete genome	2680	5361	100%	0	99%	CP001873.1
Mycoplasma gallisepticum str. R(high), complete genome	2680	5355	100%	0	99%	CP001872.1
Mycoplasma gallisepticum str. R(low), complete genome	2680	5355	100%	0	99%	AE015450.2
Mycoplasma gallisepticum strain A5969 genomic large direct repeat and flanking sequences	2680	4388	100%	0	99%	L35043.3
M.gallisepticum 16S small subunit ribosomal RNA	2680	2680	100%	0	99%	M22441.1
Mycoplasma gallisepticum strain PG31(X95) 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	2675	2675	100%	0	99%	JN935873.1

Mycoplasma gallisepticum (strain A5969) 16S-, 23S-, 5S ribosomal RNA (rrsA, rrlA, rrfA) genes	2658	2658	100%	0	99%	L08897.1
Mycoplasma gallisepticum strain ATCC 19610 16S ribosomal RNA gene, partial sequence	2641	2641	98%	0	99%	NR_104952.1
Mycoplasma gallisepticum strain NBRC 14855 16S ribosomal RNA gene, partial sequence	2627	2627	98%	0	99%	NR_113684.1
Mycoplasma imitans strain 4229 16S ribosomal RNA gene, partial sequence	2627	2627	98%	0	99%	NR_025912.1

PHENOTYPIC AND GENETIC CHARACTERIZATION OF AMOXICILLIN/CLAVULANIC ACID AND CEFOTAXIME RESISTANCE IN *SALMONELLA* MINNESOTA AND *S.* HEIDELBERG FROM BROILERS AND CARCASSES

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ABSTRACT

The objective of this study was to investigate beta-lactam antibiotics resistance of strains of *Salmonella* Minnesota and Heidelberg isolated from live broiler chickens and carcasses by Disk Diffusion Test, determination of Minimum Inhibitory Concentration (MIC) and detection of resistance genes *bla*CTX-M-8, *bla*CMY-2 and *bla*ACC-1 by PCR. There were 56 strains of *Salmonella* Minnesota and 24 of *Salmonella* Heidelberg studied that were stored in the laboratory of Avian Health - UFF. Of 80 strains analyzed, 37 strains (46.3%) were resistant to at least one antibiotic tested, 37.5% (30/80) resistant to amoxicillin/clavulanic acid and 11.3% (9/80) to cefotaxime. Determination of MIC confirmed resistance to 100% of previously tested strains by disk diffusion test. Among 30 amoxicillin/clavulanic acid resistant strains, *bla*CMY-2 gene was detected in nine, *bla*ACC-1 in six and *bla*CTX-M-8 in one strain. Among nine cefotaxime resistant strains, *bla*CTX-M-eight genes were detected in four strains and *bla*ACC-1 genes in three. The presence of cefotaxime and amoxicillin/clavulanic acid resistant *Salmonella* Minnesota and *Salmonella* Heidelberg in live chickens and carcasses and ESBL and AmpC-β-lactamases prevalence in these strains is a huge concern, because public health implications as well as the economic impact, resulting from possible restrictions on exports caused by contamination of such products.

INTRODUCTION

The consumption of poultry products, especially chicken meat, has often been associated with salmonellosis (21). *Salmonella* Heidelberg appears to be the most invasive and capable of causing diseases of greater severity than other non-typhoid serotypes, and is among the five most commonly isolated serotypes of cases of human Salmonellosis in the United States and among the three in Canada (18). Recent studies in Brazil have shown an increase in chicken infections by *Salmonella* Heidelberg and Minnesota (6, 20).

Salmonella spp. can enter the food chain from animal production, technological processing through cross-contamination and marketing of products. The use of antimicrobials in most cases of Salmonellosis in humans is not recommended, but in systemic infections such as those caused by *Salmonella* Heidelberg, the use of these drugs as third generation cephalosporins is indicated (14).

The use of antimicrobials in animal production in a therapeutic, preventive and performance-enhancing manner, especially in industrial poultry, has brought innumerable advantages, such as the reduction in feed conversion rates and the increase in productivity, which have led to a lower cost of food, hence, reduction in the rearing period to the market and a reduction in the incidence of animal diseases (9).

Bed substances in animal feed were used as a performance-enhancing additive in Brazil (4). Extended-spectrum beta-lactam resistance is usually due to the intracellular production of extended spectrum beta-lactamases (ESBL). The most frequently encountered ESBLs belong to the TEM, SHV and CTX-M groups, in which the genes encoding these characteristics are found in plasmids (5). CTX-M enzymes are able to hydrolyze third generation cell kappa (3) and are encoded by genes located in plasmids, which normally harbor other genes that confer resistance to aminoglycosides, chloramphenicol, sulfonamides, trimethoprim and tetracycline. AmpC-betalactamases are enzymes encoded by genes of chromosomal origin called ampC (13) and were also detected in *Salmonella* spp., Which has no chromosomal ampC gene, and the production of the enzyme in this bacterium is mediated by plasmid genes (17) .

The objective of this work was to characterize the phenotypic and genotypic profile of antimicrobial resistance of strains of *Salmonella* Minnesota and

Heidelberg isolated from live birds and carcasses against amoxicillin/clavulanic acid and cefotaxime.

MATERIAL AND METHODS

Samples. Eighty strains of *Salmonella enterica* were studied, being 56 Minnesota and 24 Heidelberg serotyped by the Enterobacteria Laboratory of the Department of Bacteriology of the Oswaldo Cruz Foundation in the State of Rio de Janeiro (IOC, FIOCRUZ, RJ, Brazil). These salmonellas were obtained in the years 2012 and 2013, being 19 isolates of pre-slaughter chickens and 59 isolates of slaughtered carcasses, under the Federal Inspection Service, located in the South and Center-West regions of Brazil.

Disk Diffusion Test and Minimum Inhibitory Concentration. The susceptibility of the samples to the antimicrobial agents Amoxicillin/Clavulanic acid (20/10µg) and Cefotaxime (30µg) was evaluated by the disc diffusion test according to Kirby-Bauer (2), following resistance parameters provided by CLSI (7). For the strains considered resistant, the MIC was determined by the use of Etest® - gradient technique for the determination of MIC at scale (BioMérieux, France), according to the manufacturer's instructions. The density of the bacterial suspension was adjusted to approximately 10^6 CFU/mL comparing with the 0.5 of MacFarland scale (BioMerieux, France). The resistance was analyzed following the parameters provided by CLSI (7).

Polymerase Chain Reaction (PCR). For genotypic characterization of the resistance, PCR was performed after thermal DNA extraction. Specific primer pairs were used for detection of ESBL, which confer resistance to third Generation Cephalosporins and to AmpC-betalactamases, which confer resistance to beta-lactams (except Carbapenem) and are resistant to Clavulanic Acid.

Each reaction contained 29.25 µL of water for PCR; 5.0 µL DMSO; 5.0 µL of 10X Buffer; 2.0 µL of 50mM MgCl2; 2.5 µL of each primer at 10 pmol / µL (Invitrogen); 1.0 µL of 10 mM dNTP; 0.25 µL 5U / µL Taq Polymerase (Ludwig Biotec); 2.5 µL of DNA, totaling 50 µL final volume. After previous denaturation at 94ºC for 10 minutes, 35 cycles were used, being each one of 94ºC for 30 seconds, 52ºC for 30 seconds, 72ºC for one minute, and a final extension at 72ºC for 10 minutes (15). PCR Controls used were *Salmonella* Heidelberg (IOC 200/14) (positive) and *Klebsiella pneumomiae* (CCBH 3589) (negative), both from the Enterobacteria Laboratory of the Department of Bacteriology, FIOCRUZ, RJ, Brazil.

RESULTS

Resistance to at least one antimicrobial test was observed in 46.3% (37/80) of the strains, 37.5% (30/80) resistant to Amoxicillin/Clavulanic acid and 11.3% (9/80) resistant to Cefotaxime. Of the 30 strains resistant to Amoxicillin/Clavulanic Acid, 23 were *Salmonella* Heidelberg isolated from carcasses and seven were *Salmonella* Minnesota, of which three were isolated from live chickens and four from carcasses. The nine strains resistant to Cefotaxime were *Salmonella* Minnesota, being four from live chickens and five from carcasses. Two strains were resistant to both antimicrobials, being both *Salmonella* Minnesota, one isolated from live chicken and the other from carcass.

The results obtained in the MIC determination confirmed the resistance profile found in the nine isolates resistant to Cefotaxime and in the 30 resistant to Amoxicillin/Clavulanic Acid.

Of the 30 Amoxicillin/Clavulanicid resistant strains, the *bla*CTX-M-8 gene was detected in 3.34% (1/30), the *bla*CMY-2 gene in 30% (9/30) and the *bla*ACC-1 gene in 20% (6/30). The positive strain for the *bla*CTX-M-8 gene belonged to the Minnesota serotype, isolated from live chicken. Among the nine strains positive for the *bla*CMY-2 gene, six belonged to the Minnesota serotype, three of which were isolated from live chicken and three from carcasses. And the last three strains belonged to the Heidelberg serotype isolated from carcasses. The blaACC-1 gene was found in six strains of *Salmonella* Minnesota, two of which were isolated from live chickens and four from carcasses.

Among the nine strains resistant to Cefotaxime, the *bla*CTX-M-8 gene was detected in 44.45% (4/9) and the *bla*ACC-1 gene in 33.34% (3/9). The *bla*CTX-M-8 gene was detected in four Minnesota strains, two of which were isolated from live chickens and two from carcasses. The three strains positive for the *bla*CAC-1 gene belonged to the Minnesota serotype, two of which were live chicken isolates and one of carcass.

DISCUSSION

The presence of the *bla*CMY-2 gene in *Salmonella* Heidelberg Amoxicillin/Clavulanic acid resistant strains from chickens or derived products in our study was also reported in Canada and the United States, relating it to *Salmonella* Heidelberg outbreaks in humans (1, 11). These results demonstrated, as in our study, the characteristic of AmpC-type betalactamases, which promote resistance to penicillins, cefoxitins, cephalosporins of 1st, 2nd and 3rd generations and β-lactam combinations + β-

lactamase inhibitor (Clavulanic Acid). It also shows the emergence of the gene, which might be a consequence of the use of these antimicrobials in poultry.

In this study, the presence of another gene from the AmpC beta-lactamase family, *bla*ACC-1, was also detected in some isolates resistant to Amoxicillin/Clavulanic acid and/or Cefotaxime. The presence of this gene in samples resistant to Cephalosporins and Penicillins has also been reported by other authors (12, 16).

In this study it was also found ESBL, from the CTX-M family, in samples resistant to Cefotaxime, in agreement with Silva et al. (19), by analyzing 93 strains of *Salmonella* Schwarzengrund and Agona isolated from the poultry, and Fernandes et al. (10), by studying 153 strains of *Salmonella* Typhimurium isolated from humans and other animals from São Paulo and Porto Alegre, Brazil. Detection of the CTX-M-8 gene in this study in four strains is consistent with the finding of increased hydrolytic activity on cefotaxime, as demonstrated by the values of the Disk Diffusion Test and MIC. In addition, this gene was also found in a strain sensitive to Cefotaxime, which may contribute to resistance to other antimicrobials.

With the increased prevalence of ESBL-producing bacteria, rapid and accurate identification have become increasingly important for clinical purpose. Despite the variety of methods available, the identification of ESBL by conventional phenotypic methods is difficult in the laboratory routine due to the large number of variants of betalactamases, the association of these ESBL with AmpC, or the association with metallo-betalactamases (MBL) or modification of the outer membrane permeability (8). Thus, genotypic determination has the potential to determine the resistance-causing gene and translate this into a clinically useful information that may assist in improving the diagnostic practice and treatment of Salmonellosis.

Detection of the *bla*CTX-M-8, *bla*CMY-2 and *bla*ACC-1 resistance genes in *Salmonella* Minnesota and *Salmonella* Heidelberg strains characterizes the diversity of resistance factors, and the possibility of transmission of these genes by plasmids to other serotypes or other bacterial species. The need for more comprehensive studies. Taking into account that the Heidelberg serotype plays an important role as zoonosis, and that the resistance genes detected in isolates of the Minnesota serotype can be transferred to other pathogenic salmonella serotypes in humans.

CONCLUSION

The presence of *Salmonella* Heidelberg and Minnesota serotypes resistant to Amoxicillin/Clavulanic Acid and/or Cefotaxime in live chicken and carcass evidences the importance of chicken as a potential source of transmission of these serotypes with implications for collective health.

Measures to promote awareness, regulation and research on the adequate use of antimicrobials in animal production should be implemented in order to reduce the impact on public health and increase quality of poultry carcass and by products in local and al market.

REFERENCES

1. Andrysiak, A. K., A. B. Olson, D. M. Tracz, K. Dore, R. Irwin, L. K. Ng, and M. W. Gilmour. Genetic characterization of clinical and agri-food isolates of multi drug resistant *Salmonella enterica* serovar Heidelberg from Canada. BMC microbiol. 8:89. 2008.

2. Bauer, A. W., W. M. M. Kirby, J. C. T. Sherris, and M. Turck. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493-496. 1966.

3. Bonnet, R. The Lys234Arg substitution in the enzyme SHV-72 is a determinant for resistance to clavulanic acid inhibition. Antimicrob. Agents Chemother. 52:1806-1811. 2008.

4. Brasil. Instrução Normativa SDA nº 26, de 09 de julho de 2009. Regulamento Técnico para a fabricação, o controle de qualidade, a comercialização e o emprego de produtos antimicrobianos de uso veterinário. Ministério da Agricultura, Pecuária e Abastecimento. Available from: http://www.agricultura.gov.br/arq_editor/file/CRC/IN%2026-2009%20-%20Reg%20T%C3%A9cniconormas%20fabrica%C3%A7%C3%A3o%20emprego%20antimicrobianos%20 uso%20vet.pdf. 2009.

5. Cánton, R., J. M. González-Alba, and J. Gálan. CTX-M enzymes: origin and diffusion. Front. Microbiol.3:77-95. 2010.

6. Cardoso, A., A. Kanashiro, G. Stoppa, A. Castro, R. Luciano, and E. Tessari. Ocorrência de *Salmonella* spp. em carcaças de frango provenientes de abatedouros do estado de São Paulo, Brasil, no período de 2000 a 2010. Rev. Cient. Elet. Med. Vet. 24:1-12. 2015.

7. [CLSI] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From animals; Approved Standard. 4th. VET01-A4. 33(7). 94p. 2013.

8. Drieux, L., F. Brossier, W. Sougakoff, and V. Jarlier. Phenotypic detection of extended-spectrum b-lactamase production in Enterobacteriaceae: review

and bench guide. Clin. Microbiol. Infect. 14: 90-103. 2008.

9. [EFSA] European Food Safety Authority. The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. [Internet]. EFSA Journal. 12:1-336. Available from: http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-in-zoonotic-and-indicator-bacteria-summary-report-2012.pdf . 2014.

10. Fernandes, S. A., D. L. Paterson, Â. C. Ghilardi-Rodrigues, J. M. Adams-Haduch, A. T. Tavechio, and Y. Doi. CTX-M-2–producing *Salmonella* Typhimurium isolated from pediatric patients and poultry in Brazil. Microb. Drug Resist. 15:317-321. 2009.

11. Folster, J. P., G. Pecic, A. Mccullough, R. Rickert, and J. M. Whichard. Characterization of bla CMY-encoding plasmids among *Salmonella* isolated in the United States in 2007. Foodborne Pathog. Dis. 8:1289-1294. 2011.

12. Gokul, B. N., G. A. Menezes, and B. N. Harish. Acc-1 β-lactamase–producing *Salmonella enterica* serovar Typhi, India. Emerg. Infect. Dis. 16:1170. 2010.

13. Hanson, N. D. AmpC β-lactamases: what do we need to know for the future? J. Antimicrob. Chemother. 52:2-4. 2003.

14. Hoffmann, M., S. Zhao, J. Pettengill, Y. Luo, S. R. Monday, J. Abbott, S. L. Ayers, H. N. Cinar, T. Muruvanda, C. Li, M. W. Allard, J. Whichard, J. Meng, E. W. Brown, and P. F. McDermott. Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype Heidelberg isolates from humans, retail meats, and animals. Genome Biol. Evol. 6:1046-1068. 2014.

15. Jouini, A., L. Vinué, K. B. Slama, Y. Sáenz, N. Klibi, S. Hammami, A. Boudabous, and C. Torres. Characterization of CTX-M and SHV extended-spectrum β-lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia. J. Antimicrob. Chemother. 60:1137-1141. 2007.

16. Ktari, S., G. Arlet, C. Verdet, S. Jaoua, A. Kachrid, S. B. Redjeb, F. Mahjoubi-Rhimi, and A. Hammami. Molecular epidemiology and genetic environment of acquired bla ACC-1 in *Salmonella enterica* serotype Livingstone causing a large nosocomial outbreak in Tunisia. Microb. Drug Resist. 15:279-286. 2009.

17. Pérez-Pérez, F. J., and N. D. Hanson. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. 40:2153-2162. 2002.

18. [PHAC] Public Health Agency Of Canada. *Salmonella* Heidelberg Ceftiofur-related resistance in human and retail chicken isolates. [Interenet]. [modified 2007 aug]. Available from: http://www.phac-aspc.gc.ca/cipars-picra/heidelberg/heidelberg-eng.php. 2007.

19. Silva, K. C., L. C. Fontes, A. M. Moreno, C. S. Astolfi-Ferreira, A. J. Ferreira, and N. Lincopan. Emergence of extended-spectrum-β-lactamase CTX-M-2-producing *Salmonella enterica* serovars Schwarzengrund and Agona in poultry farms. Antimicrob. Agents Chemother. 57:3458-3459. 2013.

20. Voss-Rech, D., C. S. Vaz, L. Alves, A. Coldebella, J. A. Leão, D. P. Rodrigues, and A. Back. A temporal study of *Salmonella enterica* serotypes from broiler farms in Brazil. Poult. Sci. 94:433-441. 2015.

21. [WHO] World Health Organization. *Salmonella* (non-typhoidal) - Fact sheet Nº139. [Internet]. [modified 2016 dec]. Available from: http://www.who.int/mediacentre/factsheets/fs139/en/. 2013.

GENETIC DIVERSITY OF *ORNITHOBACTERIUM RHINOTRACHEALE* ISOLATED FROM CHICKEN AND TURKEYS IN THE UNITED STATES USING MULTILOCUS SEQUENCE TYPING

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ABSTRACT

Ornithobacterium rhinotracheale (ORT), is an important bacterial pathogen of high economic concern to poultry production. This bacterium causes severe infections in chicken and turkeys worldwide. The infection caused by ORT has been responsible for huge economic losses to poultry industry by decreasing the production and increasing medication cost. Efficient diagnosis may help in reducing prevalence of the disease. The objectives of this study were to characterize ORT isolates on the basis of their geographic location, year of isolation and serotypes. A total of 47 ORT collected from different geographic locations in the USA were analyzed by Multilocus Sequence Typing (MLST). All of 47 isolates were confirmed as ORT by PCR targeting *16SrRNA* gene. Four of the isolates were excluded from the study due to bad sequences for one or more genes. The results of MLST for overall ORT isolates revealed six different sequence types (STs). ST-1was the predominated sequence type among all isolates followed by ST-9 and ST-8. Only one isolate i.e., NCF34 was identified as ST-2. No significant variation was seen in sequence types isolated from different years. On the basis of origin 25 isolates were from turkeys and 18 were from chickens. From turkeys, 76% belonged to ST-1, 12% to ST-8, 8% to ST-9 and 4% to ST-15. In Chickens 68% belonged to ST-1, 15.7% to ST-9, 5% to ST-5, ST-15 and ST-2. There was less genetic variability found in isolates from California. In total from 33 isolates, 24 were ST-1, 4 were identified as ST-9, 2 as ST-15 and 1 for both ST-5 and ST-2. Same results were observed for ORT isolates from Minnesota. The most prevalent sequence type was ST-1 (7/11), followed by ST-8 (3/11) and ST-9 (1/11).

INTRODUCTION

Respiratory diseases in poultry play an important role in huge economic losses in the form of high rate of morbidity and mortality, increased medication cost, increased condemnation rate, decreased production rate, decreased hatchability and poor egg shell quality (1). ORT is one of the well-known pathogen causing these economic losses to poultry industry. ORT is a gram negative, rod, non-spore forming, non-motile and pleomorphic organism belonging to rRNA superfamily V. This bacterium was first isolated from turkeys and chickens in early 1990s and become an important pathogen of interest (2). ORT infection is characterized tracheitis, airsacculitis, increased mortality, growth retardation, decreased egg production and fibrinous pneumonia in severely affected birds (3, 4). In North America (California) ORT was isolated for the first time from infected turkeys (5).

ORT has been detected in poultry production from many countries like South Africa, Brazil, United States of America, France, Netherland, Germany, Japan, Taiwan, Hungary and many more (1, 6). The most common route of transmission of this organism is horizontal (aerosol) transmission while vertical transmission via trans-ovarian and through cloacal contamination is also reported (7). Poor management, high stocking density, inadequate ventilation, high ammonia level, poor hygiene and litter conditions are considered to be the predisposing factors for ORT infection in flocks (8). Vaccination and adaption of preventive measures could help in control of many bacterial diseases. Temperature sensitive ORT mutant strain has been developed for use as live vaccine to control ORT infection in field (9).

ORT can usually be isolated from infected tissue i.e., lungs, trachea, air sacs, ovary and oviduct (5). Diagnosis of ORT included ELISA, PCR, and agar gel precipitation test (AGP) is well known method for serotyping of ORT. Eighteen different serotypes have been reported nowadays. Cross reaction of serotypes among each other is a big drawback and some of the strains were unable to serotypes by this method (10). The most important and common serotypes is serotype

A, which comprised of 95% of ORT strains from chicken and 50% from turkeys (7, 11). ORT A is the most frequently serotype isolated from turkeys and ORT C from chicken in the Midwest part of United States.

Various typing methods have been used for characterization of ORT strains for epidemiological investigations. Some of the techniques are; 16S ribosomal RNA (rRNA) gene analyses, repetitive sequence based polymerase, random amplified polymorphic DNA (RAPD) chain reaction (rep-PCR), multi-locus enzyme electrophoresis (MLEE) and pulsed-field gel electrophoresis (PFGE) (12-14). Although some of these methods have strong discrimination power but these techniques have limitations.

MLST is considered as a powerful tool for molecular characterization of important bacteria and fungi in epidemiological studies. In this method different housekeeping genes distributed on the whole genome of organisms are amplified by PCR and analyzed for variations. Results of MLST are portable, reproducible and unambiguous, using by most of the laboratory as a tool of epidemiological investigation (15, 16).

OBJECTIVES

The purpose of this study was to determine genetic diversity of ORT isolates on the basis of their geographic location, year of isolation and species of origin.

MATERIALS AND METHODS

Bacterial isolates. A total of 47 ORT isolates were selected for this study. These isolates were from two different states i.e., California and Minnesota. A total of 36 isolates were included from California isolated from 1998 to 2016. Four isolates from year 1998, 2001, 2003, 2006, 2009, 2012, 2014 and 2016 were from California. A total of 12 isolates were selected from Minnesota isolated from year 1996 to 2006. Information regarding geographic location, year of isolation and origin of each strain were collected and included in the study. No information about the serotypes were obtained. The bacterial isolates were revived on blood agar with 7% sheep blood containing 10µg/mL gentamycin after enrichment in tryptic soy broth (TSB) under microaerophilic condition for 48 hour at 37ºC. The characteristic ORT colonies were subjected to DNA extraction and preserved in tryptic soy broth for future record.

DNA extraction. DNA was extracted with the help of Chelex 100 resin (Cat# 1432832, Bio-Rad) according to manufacturer's instruction. Briefly, four to five identical colonies were picked with the help of sterile cotton swab and suspended in 1 mL distilled water in 1.5mL eppendorf tubes. The suspension was pelleted by centrifuging at 10000rpm for 10 minutes. The supernatant was discarded and 300 µL of 10% Chelex was added to each tube. The pellet was dissolved by vortexing and heated at 98ºC for 30 minutes in dry heating block. After centrifugation at 14000 rpm for 10 minutes, 120 µL of supernatant were collected in new tube and used for PCR reaction. The extracted DNA was stored at -20ºC until further use.

Confirmation by PCR. The suspected isolates were confirmed as ORT by PCR using *16SrRNA* primer as described by Van Empel and Hafeez 1999 (1). Amplification was carried out in 25µL reaction mixture: 12.5µL PCR mastermix (Lucigen cat#33311), 1µL of both forward and reverse primers and 8.5µL of nucleases free water. PCR conditions were set as 95˚C for 10 minutes (initial denaturation), 94˚C for one minute, 52˚C for one minute, 72˚C for 90 seconds, and 72˚C for 10 minutes (final extension) and hold at 4˚C for unlimited time.

MLST (multi locus sequence typing). MLST was initiated by selecting primers for ORT from PUBMLST website (https://pubmlst.org/orhinotracheale/). The primers optimized for seven housekeeping genes *i.e., adk* (Adenylate kinase), *aroE* (Shikimate 5-dehydrogenase), *fumC* (Fumarase, class II), *gdhA* (Glutamate dehydrogenase/ Leucine dehydrogenase), *mdh* (Malate dehydrogenase NAD), *pgi* (Glucose-6-phosphate isomerase), and *pmi* (Phosphomannose isomerase) (10). Amplification was carried out in 25µL reaction mixture: 12.5µL PCR mastermix (Lucigen cat#33311), 1µL of both forward and reverse primers and 8.5µL of nucleases free water. The amplification was carried out in T100 thermal cycler (BioRad USA) with protocol of initial denaturation at 95˚C for 10 minutes, denaturation at 94˚C for one minute, annealing at 52˚C for one minute, extension at 72˚C for 90 seconds, final extension at 72˚C for 10 minutes and hold at 4˚C for unlimited time. Nuclease free water water was included as negative control. The PCR products were visualized using 1.2% (w/v) agarose gel electrophoresis. The PCR products were cleaned up using the Qiagen PCR product purification kit (Qiagen USA), and Sanger sequencing of genes was conducted by University of Minnesota Genomic Centre (UMGC) Minnesota USA. The sequences were assembled using the assemblage implemented in Sequencher 5.4 software. All allelic sequences were queried against the ORT MLST database. Alleles already present in the database were assigned the numbers given there; novel alleles and sequence types (STs) were submitted to the ORT MLST database and assigned new numbers.

RESULTS

All of 47 isolates were confirmed as ORT by PCR targeting *16SrRNA* gene. Four of the isolates were excluded from the study due to bad sequences for one or more genes. The results of MLST for overall ORT isolates revealed six different sequence types (STs). ST-1was the predominated sequence type among all isolates followed by ST-9 and ST-8. Only one isolate i.e., NCF34 was identified as ST-2. *No significant variation was seen in sequence types isolated from different years*. On the basis of origin 25 isolates were from turkeys and 18 were from chickens. From turkeys, 76% belonged to ST-1, 12% to ST-8, 8% to ST-9 and 4% to ST-15. In Chickens 68% belonged to ST-1, 15.7% to ST-9, 5% to ST-5, ST-15 and ST-2. There was less genetic variability found in isolates from California. In total from 33 isolates, 24 were ST-1, 4 were identified as ST-9, 2 as ST-15 and 1 for both ST-5 and ST-2. Same results were observed for ORT isolates from Minnesota. The most prevalent sequence type was ST-1 (7/11), followed by ST-8 (3/11) and ST-9 (1/11).

REFERENCES

1. Van Empel, P.C., and H.M. Hafez *Ornithobacterium rhinotracheale*: A review. Avian pathology : journal of the W.V.P.A 28:217-227. 1999.

2. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, and et al. *Ornithobacterium rhinotracheale* gen. nov., sp. nov., isolated from the avian respiratory tract. International journal of systematic bacteriology 44:24-37. 1994.

3. Thachil, A.J., B.T. Velayudhan, V.C. Lopes-Berkas, D.A. Halvorson, and K.V. Nagaraja Application of polymerase chain reaction fingerprinting to differentiate *Ornithobacterium rhinotracheale* isolates. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 19:417-420. 2007.

4. Zorman-Rojs, O., I. Zdovc, D. Bencina, and I. Mrzel Infection of turkeys with *Ornithobacterium rhinotracheale* and *Mycoplasma synoviae*. Avian diseases 44:1017-1022. 2000.

5. Roepke, D.C., A. Back, D.P. Shaw, K.V. Nagaraja, S.J. Sprenger, and D.A. Halvorson Isolation and identification of *Ornithobacterium rhinotracheale* from commercial turkey flocks in the upper midwest. Avian diseases 42:219-221. 1998.

6. Tsai, H.J., and C.W. Huang Phenotypic and molecular characterization of isolates of *Ornithobacterium rhinotracheale* from chickens and pigeons in Taiwan. Avian diseases 50:502-507. 2006.

7. van Empel, P., H. van den Bosch, P. Loeffen, and P. Storm Identification and serotyping of *Ornithobacterium rhinotracheale*. Journal of clinical microbiology 35:418-421. 1997.

8. Pan, Q., A. Liu, and C. He Co-infection of *Ornithobacterium rhinotracheale* with Streptococcus zooepidemicus in chickens. Avian diseases 56:680-684. 2012.

9. Lopes, V., A. Back, D.A. Halvorson, and K.V. Nagaraja Minimization of pathologic changes in *Ornithobacterium rhinotracheale* infection in turkeys by temperature-sensitive mutant strain. Avian diseases 46:177-185. 2002.

10. Thieme, S., K. Muhldorfer, D. Luschow, and H.M. Hafez Molecular Characterization of the Recently Emerged Poultry Pathogen *Ornithobacterium rhinotracheale* by Multilocus Sequence Typing. PloS one 11:e0148158. 2016.

11. Numee, S., R. Hauck, and H.M. Hafez Detection and typing of *Ornithobacterium rhinotracheale* from German poultry flocks. Avian diseases 56:654-658. 2012.

12. Hafez, H.M., and R. Sting Investigations on different *Ornithobacterium rhinotracheale* "ORT" isolates. Avian diseases 43:1-7. 1999.

13. Amonsin, A., J.F. Wellehan, L.L. Li, P. Vandamme, C. Lindeman, M. Edman, R.A. Robinson, and V. Kapur Molecular epidemiology of *Ornithobacterium rhinotracheale*. Journal of clinical microbiology 35:2894-2898. 1997.

14. Leroy-Setrin, S., G. Flaujac, K. Thenaisy, and E. Chaslus-Dancla Genetic diversity of *Ornithobacterium rhinotracheale* strains isolated from poultry in France. Letters in applied microbiology 26:189-193. 1998.

15. Maiden, M.C. Multilocus sequence typing of bacteria. Annual review of microbiology 60:561-588. 2006.

16. Spratt, B.G. Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. Current opinion in microbiology 2:312-316. 1999.

Table 1. Primer used for ORT identification and MLST.

Gene	Primer (5´-3´)	Fragment size	Reference
16SrRNA	F: GAGAATTAATTTACGGATTAAG R: TTCGCTTGGTCTCCGAAGAT	784bp	(Van Empel & Hafeez 1999)
Adk	F: GGCAGTGGAAAAGGAACTCA R: TCTAAACTTCCTTCGCCGTTT	393bp	(Thieme *et al.* 2016)
AroE	F: GGACTCATCGGCAGAAACAT R: TGATGTTGGCATCTTGTGCT	489bp	
fumC	F: CACGCCACAAGGTTATGATG R: TAAACGCACGGCTTCTTCTT	489bp	
gdhA	F: TCTGGTAGAGCACCAAACCA R: GCTTGTTTTGCAACCACTCA	480bp	
Mdh	F: CGCGAAGAATTAATCGGAAC R: CTCTTACTTGCGCAACAGCA	519bp	
Pgi	F: AAAGCGACATTGCCAAACAT R: TTTCGAGTTCCGCTCTCACT	492bp	
Pmi	F: TGATGTGCAAGGCAATGTTT R: CTGTGTCGAGCGAAATGCTA	489bp	

Table-2: Distribution of ORT STs.

ID	Year of Isolation	Origin	Location	*adk*	*aroE*	*fumC*	*gdh*	*mdh*	*pgi*	*pmi*	ST
NCF01	1998	Chicken	California	11	1	1	1	1	1	1	**1**
NCF02	1998	Chicken	California	11	1	1	1	1	1	1	**1**
NCF03	1998	Turkey	California	1	1	1	1	1	1	1	**1**
NCF04	1998	Turkey	California	1	1	1	1	1	1	1	**1**
NCF05	2001	Chicken	California	11	1	1	1	1	1	1	**1**
NCF06	2001	Chicken	California	1	1	1	1	1	1	1	**1**
NCF07	2001	Turkey	California	11	1	1	1	1	1	1	**1**
NCF08	2001	Turkey	California	11	1	1	1	1	1	1	**1**
NCF09	2003	Chicken	California	11	1	1	1	1	1	1	**1**
NCF10	2003	Chicken	California	1	1	1	1	1	1	1	**1**
NCF11	2003	Turkey	California	13	2	1	12	10	9	6	**15**
NCF12	2003	Turkey	California	1	1	1	1	1	1	1	**1**
NCF14	2005	Chicken	California	1	1	1	1	1	1	1	**1**
NCF15	2005	Turkey	California	1	1	1	1	1	1	1	**1**
NCF16	2005	Turkey	California	1	1	1	1	1	1	1	**1**
NCF17	2007	Turkey	California	1	1	1	1	1	1	1	**1**
NCF18	2007	Chicken	California	11	1	1	1	1	1	1	**1**
NCF19	2007	Turkey	California	11	1	1	1	1	1	1	**1**
NCF20	2007	Chicken	California	11	1	1	1	1	1	1	**1**
NCF22	2009	Chicken	California	1	1	1	1	1	1	1	**1**
NCF23	2009	Turkey	California	11	1	1	1	1	1	1	**1**
NCF24	2009	Chicken	California	13	2	6	12	10	9	6	**15**
NCF26	2011	Chicken	California	11	1	1	1	1	1	5	**1**
NCF27	2011	Chicken	California	1	1	1	1	1	1	1	**1**
NCF28	2011	Turkey	California	1	1	1	1	1	1	1	**1**
NCF29	2014	Chicken	California	1	5	1	1	1	1	14	**5**
				1	5	1	1	1	1	14	**9**
NCF30	2014	Chicken	California	11	5	1	1	1	1	1	**9**
NCF31	2014	Chicken	California	1	5	1	1	1	14	1	**9**
NCF33	2016	Turkey	California	1	5	1	1	1	1	1	**9**
NCF34	2016	Chicken	California	2	2	2	2	1	2	2	**2**
NCF35	2016	Chicken	California	1	1	1	1	1	1	1	**1**
NCF36	2016	Turkey	California	1	1	1	1	1	1	1	**1**
NMN01	1996	Turkey	Minnesota	1	1	1	1	1	1	1	**1**
NMN06	1998	Turkey	Minnesota	6	8	6	7	6	7	14	**8**
NMN07	1998	Turkey	Minnesota	6	8	6	7	6	7	7	**8**
NMN08	2000	Turkey	Minnesota	1	1	1	1	1	1	1	**1**
NMN09	2000	Turkey	Minnesota	1	5	9	1	1	1	1	**9**
NMN10	2000	Turkey	Minnesota	1	1	1	1	1	1	1	**1**
NMN11	2003	Turkey	Minnesota	6	8	6	7	6	7	7	**8**
NMN12	2003	Turkey	Minnesota	11	1	1	1	1	14	1	**1**
NMN13	2003	Turkey	Minnesota	1	1	1	1	1	1	1	**1**
NMN15	2005	Turkey	Minnesota	1	1	1	1	1	1	1	**1**
NMN16	2005	Turkey	Minnesota	1	1	1	1	1	1	1	**1**

BROILER CHICK QUALITY ASSESSMENT USING THE PASGAR SCORE AT THE TIME OF HATCH

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ABSTRACT

The quality of broiler chicks is an ongoing concern for producers as well as hatcheries. Chick quality is indicative of health, storage and incubation issues as well as a predictor of overall flock performance. Alert, clean chicks with healed navels and no obvious deformities are some measures of good chicks however, these parameters are subjective. To quantify this, some methods of scoring have been developed such as the Tona or Pasgar scores. Our study looked at hatchery performance, the median Pasgar Score (chick quality) of commercial broiler chicks and broiler breeder flock production parameters (age, fertility, hatchability). We found differences in Pasgar Scores between various ages of broiler breeders even after the removal of cull chicks. As broiler breeder age increase, hatchability and fertility decreased. The number of cull chicks' increase as flock age increased. In addition to chick quality, we observed more deformed embryos and viable embryos unable to hatch from younger flocks. Unhatched eggs from older flocks had more bacterial contamination. From this study, it is suggested there is a broiler breeder age effect on chick quality, cull percentage and increased bacterial contamination in older flocks. Further studies are required to investigate the source of the bacterial contamination and its effect on broiler performance and first week mortality.

INTRODUCTION

Good quality chicks are important for broiler breeder, hatchery and broiler profitability (1-3). A good quality saleable chick is the primary objective for commercial hatcheries (1, 3). Hatchability alone does not indicate or predict overall flock performance (1). Leg deformities, thick bellies and unhealed navels can contribute to poor flock performance (2, 4), increase culling on-farm and contribute to first week mortality. Chick quality can be subjective; therefore scoring systems have been developed to alleviate this (1, 3, 4). Factors such a breeder health and age, egg storage duration and conditions and incubation parameters can all contribute to overall quality (1, 4). Poor quality chicks may be faced with more challenges on-farm resulting in higher mortality, lower weight gain and feed conversion and overall decreased flock performance (1, 5). In this study, we looked at chick quality by the Pasgar Score and conducted breakout analysis to investigate the link between chick quality and overall flock health.

MATERIALS AND METHODS

Broiler breeders. Production parameters such as strain, origin (domestic or imported), fertility and hatchability were collected for all parent flocks used in this study. Flock ages were divided into young (<34 weeks), prime (34-49 weeks) and old (>49 weeks).

Chick quality scoring. Two commercial broiler hatcheries in Western Canada were used in this study. A total of 7,978 chicks were scored using the Pasgar Score (6) after the removal of cull chicks from 138 parent flocks. Chicks were given a score out of 10, with one point deducted for deficiencies in reflex, leg and beak abnormalities, thick bellies and unhealed navels. The lowest score awarded was five.

Breakout Analysis. Unhatched eggs at day 21 of incubation were investigated by a breakout analysis. A total of 1,696 eggs were analysed and categorised as visual bacterial contamination, cracks, infertile, early dead, mid dead, late dead, pipped dead, slow or deformed.

RESULTS AND CONCLUSIONS

The average Pasgar score for chick quality was 9.23, 9.16 and 8.99 for young, prime and old flocks respectively. As expected, the average fertility was 91% for young and prime flocks and 84% for old flocks and the average hatchability was 86% for young, 84% for prime and 73% for old flocks. The percentage of cull chicks increased as flock age increase with 1.23% and 1.47% of cull chicks removed in young and prime flocks and 3.37% in old flocks. There were no notable differences between parent flock ages in regards to early and mid-dead embryos.

These findings were consistent between both hatcheries. The number of slow embryos *i.e.* viable embryos unable to hatch, was highest in young flocks. This may be due to stronger, thicker shells of young breeders and a decrease in chick strength (2, 7). There was a higher incidence of bacterial contamination in older breeder eggs which may be due to poorer shell quality. However, other factors must be investigated further. The effect of temperature on these parameters may play a role (3), such as larger eggs from older flocks producing a higher rate of heat production (4, 7). The resulting chick quality and embryo mortality may be due to egg storage conditions (8) and/or inadequate incubation conditions that do not take into account breeder age or strains (1-3) as each have varying requirements for temperature and humidity (1,2). The role of humidity and water loss (7) can also influence chick quality and performance. Unhealed navels can increase the risk of yolk sac infections and overall first week mortality (5). According to our results, we have noticed poorer chick quality scores and a higher incidence of bacterial contamination from older flocks. Controlled experimental studies are required to determine the link between chick quality and incubation conditions and their impact on first week mortality.

REFERENCES

1. Tona, K., Bruggeman, V., Onagbesan, O., Bamelis, F., Gbeassor, M., Mertens, K. and E. Decuypere. Day-old chick quality: Relationship to hatching egg quality, adequate incubation practice and prediction of broiler performance. Avian Poult. Biol. Rev. 16(2):109-119. 2005.

2. Abudabos, A. The effect of broiler breeder strain and parent flock age on hatchability and fertile hatchability. Int. J. Poult. Sci. 9(3):231-235. 2010.

3. Sozcu, A. and A. Ipek. Quality assessment chicks from different hatcher temperatures with different scoring methods and prediction of broiler growth performance. J. Appl. Anim. Res. 43(4):409-416. 2015.

4. Boerjan, M., and T. A. Scott. Incubation for uniformity. Australian Poultry Science Symposium. 18:174-181. 2006.

5. van de Ven, L.J.F., van Wagenberg, A.V., Uitdehaag, K.A., Groot Koerkamp, P.W.G., Kemp, B. and H. van den Brand. Significance of chick quality score in broiler production. Animal. 6(10):1677-1683. 2012.

6. Boerjan, M. Programs for single stage incubation and chick quality. Avian Poult. Biol. Rev. 13:237-238. 2002.

7. Tona, K., Bamelis, F., Coucke, W., Bruggeman, V. and E. Decuypere. Relationship between broiler breeder age and egg weight loss and embryonic mortality during incubation in large-scale conditions. J. Appl. Poult. Res. 10:221-227. 2001.

8. Ruiz, J. and C.A. Lunam. Effect of pre-incubation storage conditions on hatchability, chick weight at hatch and hatching time in broiler breeders. Br. Poult. Sci. 43(3), 374-383. 2002.

CAMPYLOBACTER TRANSMISSION IN COMMERCIAL POULTRY FLOCKS IN AUSTRALIA

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ABSTRACT

The objective of this study was to investigate the horizontal transmission of *Campylobacter jejuni* and *C. coli* on free-range broiler farms located in New South Wales, Australia. Fresh faecal/caecal and environmental samples were selected from broiler flocks and enriched for the presence of *Campylobacter* using ISO 10272:2006 method. The presence of *Campylobacter* varied among broiler farms and poultry age. *Campylobacter jejuni* and/or *C. coli* were detected in the environment from inside and/or outside the shed prior to placement of day old broiler chicks in two flocks. Whereas, *Campylobacter* was not detected in any broiler flocks within the first two weeks of rearing. The PCR-High-Resolution Melt (HRM) method was used to amplify the *flaA* gene of *Campylobacter* spp. The authors found that the same *C. jejuni* strain was identified in broiler flocks and the environment of the same broiler farm. The same *C. coli* strain identified from the environment prior to chick placement was consistent in the same broiler flocks. These findings confirm that *C. coli* and *C. jejuni* from the environment play an important role of *Campylobacter* transmission in broiler flocks. Further study, a larger scale epidemiological study would warrant the transmission of *Campylobacter* spp. among Australian poultry farms.

INTRODUCTION

Campylobacter jejuni infection is the leading cause of human gastrointestinal disease in many countries (1) including Australia (2). Poultry is considered a natural host of *Campylobacter* (3) and birds become colonised at an early age (4). Although multiple *Campylobacter* controls in slaughter houses have been implemented, these have not eliminated *Campylobacter* contamination (5). One strategy to improve control of *Campylobacter* would be to prevent bacterial colonisation of the chicken flocks (6). Data about transmission patterns of *Campylobacter* in Australian commercial poultry flocks are limited. Some studies have suggested horizontal transmission including drinking water, darkling beetles (7) and litter (8) as relevant sources of *Campylobacter* infections in chickens. The effective route of *Campylobacter* transmission on poultry farms remain unclear. To date there is no effective method to control *Campylobacter* colonisation in poultry. Of further note, poultry farming practices and climatic conditions in Australia are different from those of other countries. As a result using overseas data to develop methods of *Campylobacter* control in Australia is not likely to be effective. Hence, this study aimed to investigate the horizontal transmissions of *C. jejuni* and *C. coli* among commercial poultry flocks in Australia. Data obtained in this study could enhance the knowledge of *Campylobacter* transmissions and aid development of strategies for campylobacter control and consequently benefit consumers and the poultry industry.

MATERIALS AND METHODS

Study design. Three free-range broiler farms located in New South Wales, Australia, were assessed. One target broiler flock and two adjacent flocks from each broiler farm were selected for sample collection. For example, broiler farm 1 comprised shed D (adjacent), E (target) and F (adjacent). This collection method was employed for farm 2 (G, H, I) and 3 (M, N, O).

Broiler flocks. Fresh faecal/caecal droppings (n=35 for target flock, n=10 for adjacent flocks) and environment (n=244) were randomly selected at seven day intervals starting from the day of house disinfection to first detection of *Campylobacter* in a target flock. Total nine broiler flocks were selected.

***Campylobacter* isolation**. All samples were managed with an ISO 10272:2006 method for *Campylobacter* isolation. Presumptive colonies showing typical morphological characteristics of *Campylobacter* spp. were selected to confirm the species level.

***Campylobacter* identification**. Matrix Assisted Laser Desorption Ionization Time-of-Flight or MALDI-TOF (VITEK® MS) (Biomerieux, Australia) was used to identify *C. jejuni* and *C. coli*.

Genomic DNA extraction. Purification of genomic DNA was performed using PrepMan® Ultra Sample Preparation (Applied Biosystems, Australia) according to the manufacturer's instructions. The dilution of extracted DNA samples was 1:100 by volume and stored at 4°C.

PCR-HRM. All *C. jejuni* and *C. coli* isolates were tested with the HRM and ScreenCLust analysis. The PCR-HRM protocol of *flaA* amplification was processed with a previous method using a Rotor-Gene Q with HRM data analysed using ScreenClust HRM software (9).

DNA sequencing. Representative isolates of *Campylobacter* were examined with DNA sequencing to confirm the *flaA* gene. The *flaA* PCR products were commercially sequenced (Sanger sequencing method) at the Australian Genomic Research Facility, Sydney, Australia.

RESULTS

Broiler Farm 1 (D, E, F). In Flock D (n=10), two strains of *C. jejuni* were identified with the HRM and ScreenClust analysis. The *C. jejuni* isolated from the outside of the shed (n=1) was the same strain with eight isolates collected from fresh faecal droppings. The other one fresh faecal dropping displayed a different strain. The DNA sequencing results confirmed the findings of the HRM and ScreenClust analysis and indicated that the outside of the shed and the broilers shared the same genotype of *C. jejuni*. Similarly, two strains of *C. coli* (n=8) were detected with the HRM, ScreenClust analysis and DNA sequence (data not shown). In Flock E, the HRM and ScreenClust analysis identified two strains of *C. jejuni* in a total of 42 samples isolated from the environment and the flock. An isolate collected from the faecal sample had a different HRM pattern with a high Ct value and was classified into a separate cluster, suggesting it is a different strain. However, the isolates of both strains showed a consistent *flaA* gene sequence (data not shown). This indicated that all *C. jejuni* isolates of this broiler flock and the environment were highly likely to be the same strain. All *C. jejuni* isolates (n=20) collected from Flock F belonged to the same strain according to the outcomes of the HRM and ScreenClust analysis. The DNA sequence also confirmed the results (data not shown). Nevertheless, a *C. coli* sample isolated from the outside of the shed had a HRM pattern and a *flaA* sequence different from those of other *C. coli* samples in this broiler flock (data not shown).

Broiler Farm 2 (G, H, I). *C. jejuni* samples isolated from the Flock G (n=10) belonged to two different groups based on the outcomes of the HRM and ScreenClust analysis. These were also distinguished into two *flaA* genotypes with DNA sequencing. The isolate collected from the outside of the shed shared the same genotype with that of the broiler flock. On the other hand, five different patterns of *C. jejuni* were found in isolates collected from the environment and Flock H (n=46), as per the outcomes of the HRM analysis. DNA sequencing results also showed five different *flaA* genotypes of *C. jejuni* (data not shown). As for Flock I (n=11), two HRM patterns of *C. jejuni* were identified. Results of the ScreenClust analysis and DNA sequencing confirmed this finding.

Broiler Farm 3 (M, N, O). All *C. jejuni* isolates collected from the environment and Flock M (n=11) had a consistent HRM pattern. Outcomes of the ScreenClust analysis and the DNA sequencing confirmed this (data not shown). Similarly, a *C. coli* sample isolated from outside of shed M before chick placement and a fresh faecal/caecal dropping on 24-day-olds belonged to the same strain, according to the outcomes of the HRM and DNA sequencing methods (data not shown). In Flock N, three strains of *C. jejuni* were detected in isolates (n=40) collected from the broiler flock, based on the outcomes of the HRM analysis, ScreenClust and DNA sequencing. Furthermore, two strains of *C. coli* were identified in the environment (n=3) according to the results of those tests. The result of the HRM analysis showed that all *C. jejuni* isolates (n=11) collected from the outside of the shed and Broiler Flock O had two different patterns. The HRM pattern of *C. jejuni* isolated from the outside of the shed was different from that of Flock O. Outcomes of the ScreenClust analysis and DNA sequencing confirmed the findings (data not shown). Two strains of *C. coli* were identified as per results of the HRM, ScreenClust analysis and DNA sequencing.

DISCUSSION

Campylobacter jejuni and/or *C. coli* were isolated from broiler farms in this study. On the three broiler farms studied, the free-range period was started from the third week after chick placement until the end of rearing period. Under this production system all chickens are exposed to the external environment and therefore the environment would be expected to be an important factor for *Campylobacter* transmission (10).

Campylobacter coli or *C. jejuni* was found in environment (outside) after the shed disinfection period. This indicated that the carryover of *Campylobacter* from previous flock could be a source of contamination and/or the disinfection step was improperly implemented on the broiler farms. During the rearing period, the environment including rodent faeces, floors, anteroom, wall, boots and soil were potential sources of *Campylobacter* transmission on the broiler farms in this study. The same *C. jejuni*

and/or *C. coli* strain was found among broiler flocks and the environment on the same farm. These results suggest that horizontal transmission was the most important pathway for the colonisation of the broiler flocks in this study. Thus, improved on-farm biosecurity could be used to prevent horizontal transmission on these broiler farms. Further studies are required to determine which of these sources of *Campylobacter* transmission are the most important.

CONCLUSIONS

Our findings revealed that horizontal transmission is an effective route of *C. jejuni* and *C. coli transmission* on poultry farms in Australia. The environment including outside of shed, boot outside shed, anteroom and rodent faeces ware found to be potential sources of *Campylobacter* transmission among broiler flocks. Further investigations which include more poultry farms and longitudinal studies are indicated.

REFERENCES

1. European Centre for Disease Prevention and Control [ECDC]. Annual epidemiological report on communicable diseases 2010. [Internet]. [modified 2010; cited 2017 Jan 05]. Available from: http://www.ecdc.europa.eu/en/publications/Publications/1011_SUR_Annual_Epidemiological_Report_on_Communicable_Diseases_in_Europe.pdf. 2010.

2. NNDSS. Australia's notifiable disease status, 2012: Annual report of the National Notifiable Diseases Surveillance System. Commun. Dis. Intell. 39: 46–136. 2015.

3. Hermans, D., K. Van Deun, A. Martel, F. Van Immerseel, W. Messens, M. Heyndrickx, F. Haesebrouck, and F. Pasmans, (2011). Colonization factors of Campylobacter jejuni in the chicken gut. Vet. Res. 42: 1-14. 2011.

4. Ingresa-Capaccioni, S., S. Gonzalez-Bodi, E. Jimenez-Trigos, F. Marco-Jimenez, P. Catala, S. Vega, and C. Marin. Comparison of different sampling types across the rearing period in broiler flocks for isolation of Campylobacter spp. Poul. Sci. 94: 766-771. 2015.

5. Guerin, M. T., C. Sir, J. M. Sargeant, L. Waddell, A. M. O'Connor, R. W. Wills, R. H. Bailey, and J. A. Byrd, The change in prevalence of *Campylobacter* on chicken carcasses during processing: a systematic review. Poult Sci, 89: 1070-1084. 2010

6. Newell, D. G., K. T. Elvers, D. Dopfer, I. Hansson, P. Jones, S. James, J. Gittins, N. J. Stern, R. Davies, I. Connerton, D. Pearson, G. Salvat, and V. M. Allen. Biosecurity-based interventions and strategies to reduce Campylobacter spp. on poultry farms. Appl. Environ. Microbiol. 77: 8605-8614. 2011.

7. Miflin, J. K., J. M. Templeton, and S. J. More. Epidemiological Studies of Campylobacter colonization of broiler flocks in South East Queensland. Proceeding Australian Poultry Science Symposium. 140-143. 2001

8. Shanker, S., A. Lee, and T.C. Sorrell. Horizontal transmission of *Campylobacter jejuni* amongst broiler chicks: experimental studies. Epidemiol. Infect. 104: 101-110. 1990.

9. Merchant-Patel, S., Blackall, P. J., Templeton, J., Price, E. P., Tong, S. Y., Huygens, F., and P.M. Giffard. *Campylobacter jejuni* and *Campylobacter coli* genotyping by high-resolution melting analysis of a *flaA* fragment. Appl. Environ. Microbiol. 76: 493-499. 2010.

10. Newell, D. G., and C. Fearnley., C. Sources of *Campylobacter* Colonization in Broiler Chickens. Appl. Environ. Microbiol. 69: 4343-4351. 2003.

DETECTION OF CEVAC IBRON® VACCINE AFTER BROILER HATCHERY VACCINATION

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SUMMARY

Mass application of IBV live attenuated vaccine is a preferred method for vaccination of young chicks at the broiler hatchery. Even though the mass spray vaccination is cost-effective and convenient, it does not always guarantee uniform and successful vaccine delivery. Therefore monitoring of the vaccine administration in the birds is necessary to ensure the adequate performance of vaccine application. Traditionally, green or blue dyes are added to vaccine diluents prior to vaccination and the colored tongue of the bird is considered as indication of vaccine take. However, the ingestion of vaccine solution cannot directly indicate proper vaccination. For successful vaccine outcome, vaccine virus should replicate in the trachea between three to seven days post vaccination, consequently inducing a proper immune response. Therefore detection of virus replication in vaccinated birds using virus isolation or molecular methods provide a better prediction of vaccination. With the launch of a new IBV GA08 vaccine (Cevac IBron®), a vaccine detection monitoring protocol utilizing IBV genotype specific realtime RT-PCR assays was developed to aid hatchery vaccination by monitoring the replication of vaccine virus. Detailed protocol and vaccine detection data will be discussed.

MATERIALS AND METHODS

To evaluate the detection of Cevac IBron vaccine at vaccinated broiler chicks, a detection protocol was established. The protocol parameters are as follows.

Sample size. 15 birds per flock was a recommended sample size. According to De Wit (2), 2~8 samples per flock (flock size between 100~100,000) is a required sample size when IBV prevalence is 50~100 percent.

Sample type. Trachea tissue and choanal (oropharyngeal) swabs in transport media were both tested and compared.

Sample collection time point. Generally, IBV virus starts to replicate within 24 hrs of vaccination, reaching to its peak titer at 7 to 10 days post vaccination. In the protocol, sampling at 5 days post hatchery vaccination is utilized to reduce the potential detection of positives caused by bird to bird transmission of the vaccine.

IBV vaccine detection method. Viral RNA was extracted using Mag-Bind Omega Blood and Tissue kit (OMEGA bio-tek, M6399-01) and processed for IBV realtime RT-PCR assays. Cevac IBron specific primers and probe were designed to target its unique S1 sequences. In addition to the IBron specific assay, the following IBV assays were used. Universal IBV(5'UTR)(1), Ark type (3), Massachusetts type, Connecticut type, DE072/GA98 type (4), and GA08 (unpublished data. Dr. Brian Jordan from PDRC, UGA kindly shared the information).

In the protocol evaluation, a total of 285 samples were analyzed. Samples from various sources were collected over a 6 month period and shipped to Ceva SSIU Lab Services.

RESULTS AND DISCUSSION

Cevac IBron specific realtime RT-PCR assay. IBron S1 sequences were aligned with other GA08 type virus sequences to find unique sequences in S1 gene. Primers and probe were designed targeting these unique sequences and blasted to the NCBI database. The specificity of the IBron specific assay was verified using other IBV including Ark-DPI, commercial GA08 vaccines, GA08 challenge virus. The standard curve for CEVAC IBron specific assay was plotted using mean C_T values (± standard deviation) obtained from triplicate runs using a 10-fold serially diluted IBron RNA. ($y= -3.20x + 17.861$, $R^2=0.99$, x = dilution factor, y= C_T value, Efficiency = 105.35%)

Detection of IBron. Differences of IBron and IBV vaccine detection level was looked at in different sample types, trachea and choanal swab. 30 trachea samples and 30 choanal samples were collected from two flocks at 5 days post hatchery vaccination. Chicks were vaccinated with a full dose IBron, a half dose of Ark-DPI, and a half dose of Mass type vaccine using a hatchery spray cabinet. The results are depicted in Figure 1. Choanal swabs exhibited better detection percentage overall for all realtime RT-PCR assays (including the 5'UTR assay). IBron detection level was 83% from choanal swab samples, while 63% in trachea. Mass type vaccine detection was 97% in choanl swabs and 80% in trachea samples. Detection of Ark-DPI vaccine was low as expected in both samples, however choanal swabs shows 23% positives while tracheal samples shows only 7% positives.

Results of IBron detection at five days post vaccination are shown in Figure 2. 15 choanal swabs were collected from each flock (a total of 16 flocks) at five days post hatchery vaccination. All flocks are vaccinated with a full dose of IBron. Flocks vaccinated only with IBron include flocks 3, 4, 7, 8, 11, 12, 13 and 14. In other flocks, either Ark, DE072, or Mass were combined with IBron. For IBron, above 70% of positive detection was considered good vaccine detection (dotted line in the Figure 2). With more than 70% vaccine positive birds in the flock at five days post vaccination, we expect the vaccine percent positive at seven to ten days post vaccination would reach above 80~90%, and potentially provide optimum flock immunity. All the samples were tested with the 5'UTR assay and IBron specific assay. Flocks with additional vaccines were subjected to additional assays (data not shown). In Figure 2, only IBron detection assay results are depicted. Most of the tested flocks exhibited above 70% of IBron positive detection. There were slight differences in the limit of detection of 5'UTR assay and IBron assay. Two flocks (flocks 13 and 14), vaccinated only with IBron exhibited less than 70% of IBron detection. However, some of IBron negative samples (C_T >40) were detected as low positives (C_T 37~39) by the 5'UTR assay resulting 87% and 73% positives respectively (data not shown).

The evaluation of IBron vaccination using IBron specific realtime RT-PCR assays confirmed the successful administration of the vaccine and provided the general trend of IBron vaccine detection level. Extended from this evaluation, further investigation to understand the correlation between IBron vaccine detection level at five days post vaccination and protection level against the challenge would be necessary.

REFERENCES

1. Callison, S.A., D.A. Hilt, T.O. Boynton, B.F. Sample, R. Robison, D.E. Swayne and M.W. Jackwood. Development and evaluation of a realtime Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of Virological Methods 138:60-65. 2006

2. De Wit, J.J. Detection of infectious bronchitis virus. Avian Pathol. 29:71-93. 2000

3. Roh, H.J., D.A. Hilt, S.M. Williams, and M.W. Jackwood. Evaluation of infectious bronchitis virus Arkansas-type vaccine failure in commercial broilers. Avian Dis 57(2):248-259. 2013

4. Roh, H.J., B.J.Jordan, D.A.Hilt, and M.W.Jackwood. Detection of infectious bronchitis virus with the use of real-time quantitative reverse transcriptase-PCR and correlation with virus detection in embryonated eggs. Avian Dis 58:398-403. 2014

Figure 1. Comparison results of IBV detection levels at 5 days post vaccination from trachea samples and choanal swabs. 30 trachea and 30 choanal swabs collected from two flocks vaccinated with IBron, Ark and Mass type IBV. 5'UTR: universal IBV detection assay, IBron: IBron specific assay, Ark: Ark type specific assay, Mass: Massachusetts type specific assay.

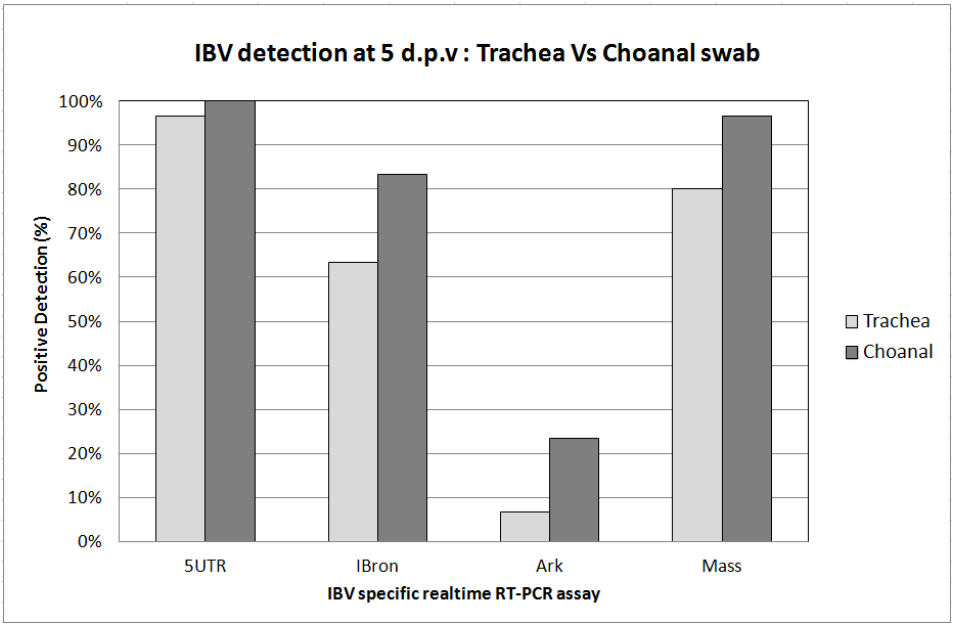
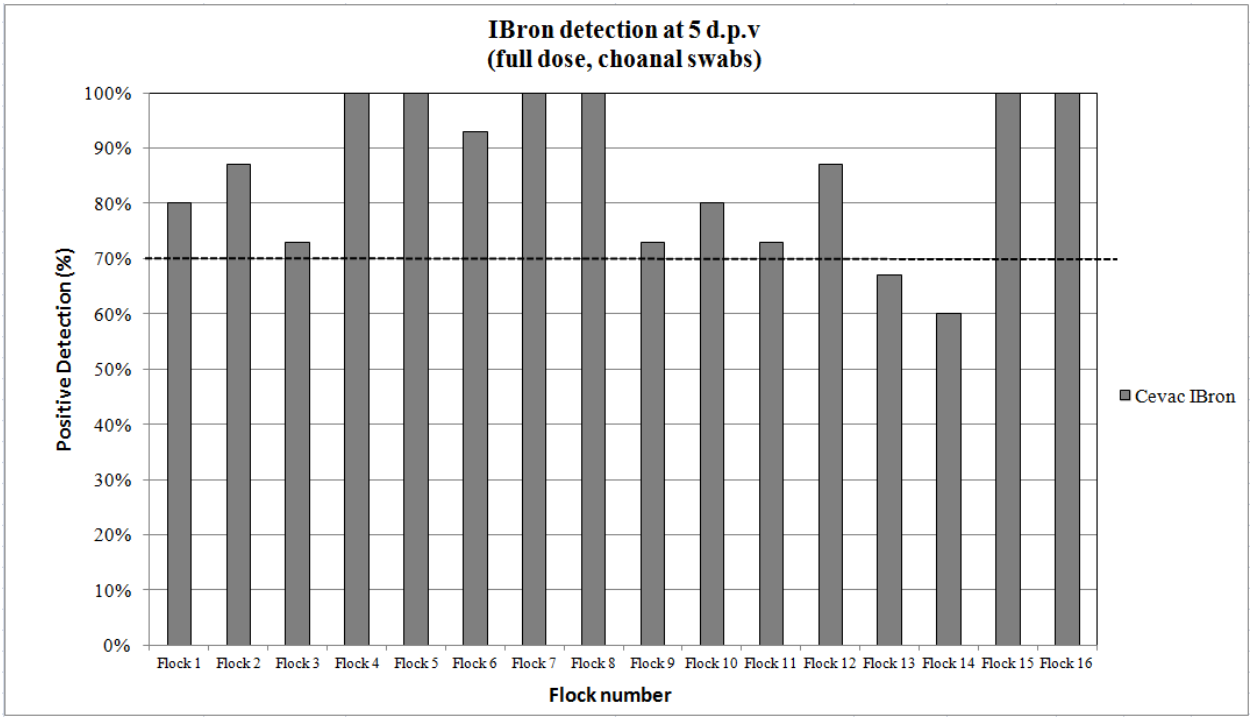


Figure 2. Detection of IBron from choanal swabs. Samples were collected at 5 days post hatchery vaccination. The results obtained by IBron specific assay are shown.



A MULTI-YEAR ANALYSIS OF AVIAN ADENOVIRUSES FROM CLINICAL CASES OF IBH

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SUMMARY

Inclusion body hepatitis (IBH) is an important disease of poultry caused by avian adenoviruses belonging to the genus *Aviadenovirus* (formerly identified as group 1). Outbreaks of IBH occur primarily in broilers aged three to eight weeks. Clinical signs include a sudden onset of mortality and occasionally visible foci on the liver. While some adenoviruses can act as primary pathogens in IBH (1), immunosuppression with infectious bursal disease virus (IBDV) and chicken anemia virus (CAV), intensifies the severity of disease. Within the *Aviadenovirus* genus, five species designated FAV group A-E contain 12 recognized serotypes based on neutralization assays, each containing representative strains. Serotyping of avian adenoviruses has largely been replaced by genotyping which is based on the genome sequence. ICTV classification of the serotypes provides a common international identification system that can circumvent confusion between the former use of US and European nomenclature.

Clinical diagnosis of the disease can be confirmed by histopathology, virus isolation and/or PCR of affected livers. Histological evaluation of affected livers reveals large intranuclear inclusion bodies which are indicative of adenovirus infection. Avian adenoviruses are easily isolated in embryos, resulting in mortality, stunting or hemorrhage, and/or in epithelial cell cultures resulting in a characteristic cytopathic effect. Adenovirus DNA can also be directly amplified from livers and sequenced for comparison of nucleotides with reference strains.

In this study, IBH clinical case submissions to the Poultry Diagnostic and Research Center at the University of Georgia from 2010-2016 were analyzed to determine the prevalence of adenovirus genotypes. For domestic cases, livers were submitted to the laboratory for virus isolation, PCR and sequencing. Virus isolation was performed by inoculating a filtered liver homogenate into confluent monolayers of primary chicken embryo liver cells prepared from 15-day-old SPF embryos. Samples positive for adenovirus exhibited the characteristic CPE 72-96 hours post inoculation. For international cases, FTA cards with liver impressions were submitted. Genotyping of domestic and international samples was performed by extracting DNA from the cell culture passages of virus isolation cases, or, from the FTA card, followed by PCR amplification of a 900 base pair region of the hexon gene including the L1 loop (2) and sequenced. Multiple alignments of the hexon product nucleotide sequences were performed using ClustalW in MEGALIGN (DNASTAR, Lasergene 14) followed by phylogenetic analysis.

An increase in the number of domestic clinical cases of IBH was observed in 2013 and 2014 compared to other years. Predominant IBH adenoviruses from US cases during 2011-2014 were serotypes 7 and 11, followed by 8b, then 8a. In 2015 and 2016, there was a significant shift and a majority of the cases were serotype 7, representing 50-80% of the isolations respectively. The remaining cases belonged to serotypes 8a, 8b and 11. The age range of broilers from the serotype 7 cases was between 2.5-5.2 weeks of age.

Clinical IBH in other parts of the world can be complicated with hydropericardium syndrome (HPS) and clear delineation of one disease or the other is not always apparent from the history provided with the case submission, thus HPS cases were also included in this analysis. Serotype 4 adenoviruses are associated with HPS (3) and were detected in a majority of the international submissions in 2011 and 2013. In 2012, serotype 11 was most commonly detected in IBH cases. In 2014, serotypes 4 and 11 were detected in equal proportions followed by 8b. As observed in the US during 2015-2016, an increase in serotype 7 adenoviruses from IBH cases was observed in over 50% of the cases submitted to PDRC.

It's not clear why there was an increase in the isolation or detection of adenovirus serotype 7 from clinical cases of IBH and whether or not there was an association with immunosuppression. In the US, some companies are electing to utilize isolates of serotype 7 in their autogenous vaccines.

REFERENCES

1. Gomis, S., A.R. Goodhope, A.D. Ojkic, and P. Willson. Inclusion body hepatitis as a primary disease

in broilers in Saskatchewan, Canada. Avian Dis, 50: 550-555, 2006.

2. Meulemans, G, M. Boschmans, T.P. van den Berg and M. Decaesstecker. Polymerase chain reaction combined with restriction enzyme analysis for detection and differentiation of fowl adenoviruses. Avian Path, 30: 355-660, 2010.

3. Ganesh, K and R. Raghavan. Hydropericardium hepatitis syndrome of broiler poultry: current status of research. Res Vet Sci, 68: 201-206, 2000.

POULTRY DISEASE DIAGNOSTIC SERVICES IN CALIFORNIA

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California has a large poultry industry located in the central San Joaquin valley, southern California and some in the north central coast. California produces annually about 300 million broilers, 12 million egg laying chickens, 15 million turkeys and about 3 million ducks. California also has a large game bird and squab industry. Four laboratories of the California Animal and Health and Food Safety Laboratory System strategically located in Turlock, Tulare, San Bernardino and Davis provide quality and timely services to the clients. These services include postmortem examinations, serology, biotechnology, FA, bacteriology, mycology, virology, histopathology, immunohistochemistry, electron microscopy and toxicology and nutritional analysis. The results are communicated to the clients promptly through oral, e-mail, fax and occasionally by land mail. The mission of the laboratory system and its interaction with the California Department of Food and Agriculture, the School of Veterinary Medicine and the clients will be presented and discussed.

PATHOLOGY OF VARIANT REOVIRUS INFECTION IN CALIFORNIA BROILER CHICKENS

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Between mid-2015 and the end of 2016, numerous cases of broiler chickens ranging in age from 16 to 40 days were submitted to the Tulare branch of the CAHFS laboratory system with a history of leg problems such as birds being down on legs, unable to walk, deviation of one or both legs laterally or anteriorly or posteriorly, uniformity issues, increased morbidity and culling. Necropsy of the chickens revealed mild to severe swelling of the hock joints due to the presence of pale yellow exudate and swollen tarsometatarsal bones. None of the birds had rupture of the tendons. A number of birds also had hydropericardium and small pale nodules on the pericardium. Histopathology revealed mild to severe lymphoplasmacytic tenosynovitis and epicarditis with lymphoid nodule formations. Occasional bird had necrosis in the liver. Reoviruses were isolated from the tendons from most cases and heart and were molecularly characterized as variants of S1133.

FEATHER FOLLICULITIS ASSOCIATED WITH FUNGUS, *ALTERNARIA* SPP. AND HIGH CONDEMNATION IN COMMERICAL PEKIN DUCKS

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Condemnation of carcasses ranging from 10 % to 20 % occurred in a flock of 20,000, 43-day-old Pekin ducks due to skin lesions. Examination of 12 processed carcasses revealed a few too many circumscribed or irregular foci of redness measuring about three to five mm in diameter scattered on the skin overlying the breast muscles. In addition, there were small pale grey soft nodules about three mm in diameter associated with feather follicles. Histopathology of the skin revealed severe fibrinosuppurative inflammation of the feather follicles with mild extension in to the dermis. Special stains such as PAS and GMS identified mycelia measuring five um in diameter and had parallel walls. The mycelia were branching with occasional septate hyphae. DNA extraction from the fungus and PCR amplification using universal primers and sequence of the amplicons revealed that the fungus was > 99 % similar to *Alternaria* spp. Damp litter, increased humidity and poor ventilation predisposed the ducks for the skin infection with the fungus. *Alternaria* spp. are ubiquitous saprophytic fungi that are present in the environment, soil, on the plants and normal skin.

DEVELOPMENT OF A RAPID QUARTZ CRYSTAL MICROBALANCE-BASED PATHOGENIC *SALMONELLA* SEROVAR DETECTION SYSTEM: PRELIMINARY LABORATORY RESULTS

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ABSTRACT

Salmonella detection on grow-out farms and processing farms is primarily done via qualitative tests that take several days (i.e. culture, enrichment and PCR). In order to reduce the time to detection and offer the ability to quantify *Salmonella* spp., we are developing a quartz crystal microbalance (QCM) based immunoassay test system for rapid detection of food-borne pathogens with a focus on *Salmonella* Enteritidis (SE) and Heidelberg (SH) detection. Initial benchtop experiments have shown detection of SE and SH at concentrations ranging from 107 to 101 CFU/mL within 10 seconds. Test solutions to date have been laboratory buffer systems as well as bacteriological culture solutions. Work is on-going to expand use in various food-based matrices.

INTRODUCTION

Detection of non-typhoidal *Salmonella* in poultry processing plants in the United States is primarily done via tests that take a minimum of 24 hours and are labor intensive (i.e. culture plus PCR or ELISA). Hence, since most poultry meat is sold within days, actionable results are typically only available on a *post hoc* basis leading to recalls and outbreaks. Furthermore, in the current system, no quantitative information with respect to the *Salmonella* load is acquired. Specifically, the current surveillance testing required by the USDA Food Safety Inspection Service (FSIS) is only qualitative and hence *Salmonella* prevalence data from the "post-chill line" (e.g. the step right before packaging) only provides information about positivity or negativity and not the actual load of *Salmonella* present in poultry meat about to be packaged. This is especially troublesome since the FSIS currently requires only one sample per week and allows up to 7.5% positive post-chill *Salmonella* in a poultry processing plant per year as part of their current Performance Standard (1). Therefore, having a practical, repeatable, rapid quantitative approach that quantifies pathogenic *Salmonella* serotypes at a sensitivity of at least 102 organisms per sample (e.g. m Lof post-chiller sample) would transform our current ability to surveil raw poultry for *Salmonella* and other pathogenic organisms. To this point, the FSIS 2017-2021 Strategic Plan outlines a number of objectives to improve food safety inspection with a focus on better testing methodologies and improved sampling rates (2).

QCM utilizes piezoelectric biosensors that detect resonance frequency changes that result from mass changes. The recorded frequency change is proportional to the concentration of the targeted analyte. Over the last 5-10 years knowledge of the rapidity and sensitivity of QCM has increased interest in utilizing this method in food systems for pathogen detection (4, 5, 6). Using QCM with an immunoassay based chemistry, recent literature has shown limits of detection (LOD) for bacteria including *E. coli* and *Salmonella* Typhimurium between 10-20 CFU mL-L (4, 6). In contrast traditional ELISA based method typically have LODs between 3-5 log CFU mL-L (7, 8). While PCR can provide more sensitive results between 1-3 log CFU mL-L the presence of inhibitors in many food and environmental matrices limit direct PCR as opposed to pre-enrichment followed by PCR (9).

In order to reduce the time to detection and offer the ability to quantify *Salmonella* spp., we are developing a QCM based immunoassay test system for rapid detection of food-borne pathogens with a focus on SE and SH detection. The QCM-based system we are developing is different from other versions in that it uses a protein-based thick film with a statically administered liquid sample instead of dynamically administered test solutions in a flow cell passed over thin monolayers. In the food industry, this technology will enable more testing of products during the entire production process to enhance food safety from infectious agents. Testing and results on the spot would be enabled for testing of irrigation water in the field, rinsates from products, etc. Due to time constraints the current testing procedure usually results in product recalls once a food related outbreak

is identified, followed by retrospective analysis to find the source of the infection.

MATERIALS AND METHODS

The detection surfaces consist of relatively large quantities of both Protein A or G and antibody in a low ionic strength buffer system at neutral pH and dried down onto the crystal surface. There is an excess of both needed to detect an analyte and generate a change in the crystal frequency, normally on the order of several hundred to several thousand hertz. In the process two populations of Protein A or G-antibody conjugates are created: one as a layer bound to the surface of the crystal and one unbound acting as a freely available pre-polymer. When a controlled amount of test solution (usually 100 uL) is aliquoted onto the coated surface the binary film rehydrates. If the target analyte is present it will bind to both populations of the detection conjugates, creating a 3-dimensional complex which is detectable by a change in the baseline frequency of the coated crystal (Figure 1). The change in frequency from baseline is proportional to the concentration of the analyte. The reaction is detected in real time as it occurs by monitoring the change in frequency. The reaction occurs within 5-10 seconds and is stable within 20-30 seconds (See Figure 1). Due to crosslinking with the antigen of interest the change in frequency signal is amplified an order of magnitude greater than current QCM test systems.

RESULTS AND CONCLUSIONS

Initial benchtop experiments have shown detection of SE and SH at concentrations ranging from 107 to 101 CFU/mL in tryptic soy broth media (Figure 2). Interference studies demonstrated detection of ST in the presence of 105 CFU/mL each of *E. coli* and *C. freundii* in a meat matrix/growth media suspension (Figure 2). Results were available within 10 seconds of aliquoting liquid samples onto coated crystals. Further updated data will be presented.

REFERENCES

1. CDC Salmonella Page [https://www.cdc.gov/salmonella/]
2. USDA-FSIS: Pathogen Reduction - Salmonella and Campylobacter Peformance Standards Verification Testing FSIS ed.2016.
3. FSIS: FSIS Strategic Plan 2017-2021. pp. 642016:64.
4. Guo X, Lin C-S, Chen S-H, Ye R, Wu VCH: A piezoelectric immunosensor for specific capture and enrichment of viable pathogens by quartz crystal microbalance sensor, followed by detection with antibody-functionalized gold nanoparticles. Biosensors & Bioelectronics 2012, 38:177-183.
5. Masdor NA, Altintas Z, Tothill IE: Sensitive detection of Campylobacter jejuni using nanoparticles enhanced QCM sensor. Biosensors & Bioelectronics 2016, 78:328-336.
6. Salam F, Uludag Y, Tothill IE: Real-time and sensitive detection of Salmonella Typhimurium using an automated quartz crystal microbalance (QCM) instrument with nanoparticles amplification. Talanta 2013, 115:761-767.
7. Park S, Kim H, Paek S-H, Hong JW, Kim Y-K: Enzyme-linked immuno-strip biosensor to detect Escherichia coli O157 : H7. Ultramicroscopy 2008, 108:1348-1351.
8. Wang N, He M, Shi H-C: Novel indirect enzyme-linked immunosorbent assay (ELISA) method to detect Total E. coli in water environment. Analytica Chimica Acta 2007, 590:224-231.
9. Charlton BR, Walker RL, Kinde H, Bauer CR, Channing-Santiago SE, Farver TB: Comparison of a Salmonella Enteritidis-specific polymerase chain reaction assay to delayed secondary enrichment culture for the detection of Salmonella Enteritidis in environmental drag swab samples. Avian Diseases 2005, 49:418-422.

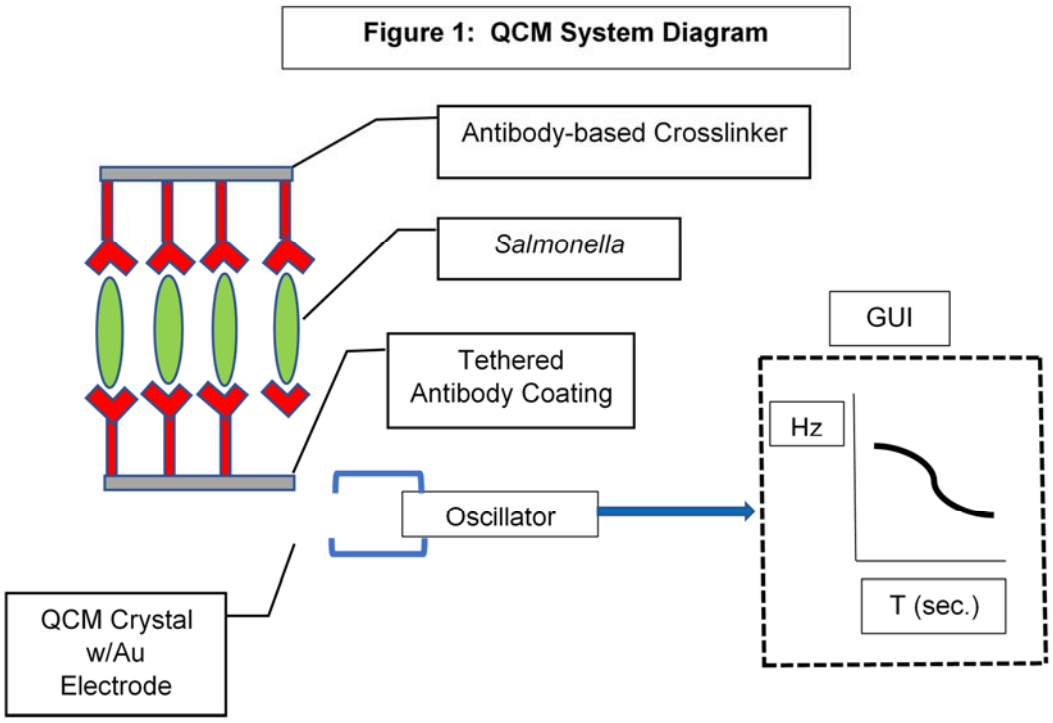
Figure 1: QCM System Diagram
Antibody-based Crosslinker
Salmonella
Tethered Antibody Coating
Oscillator
QCM Crystal w/Au Electrode
GUI
Hz
T (sec.)

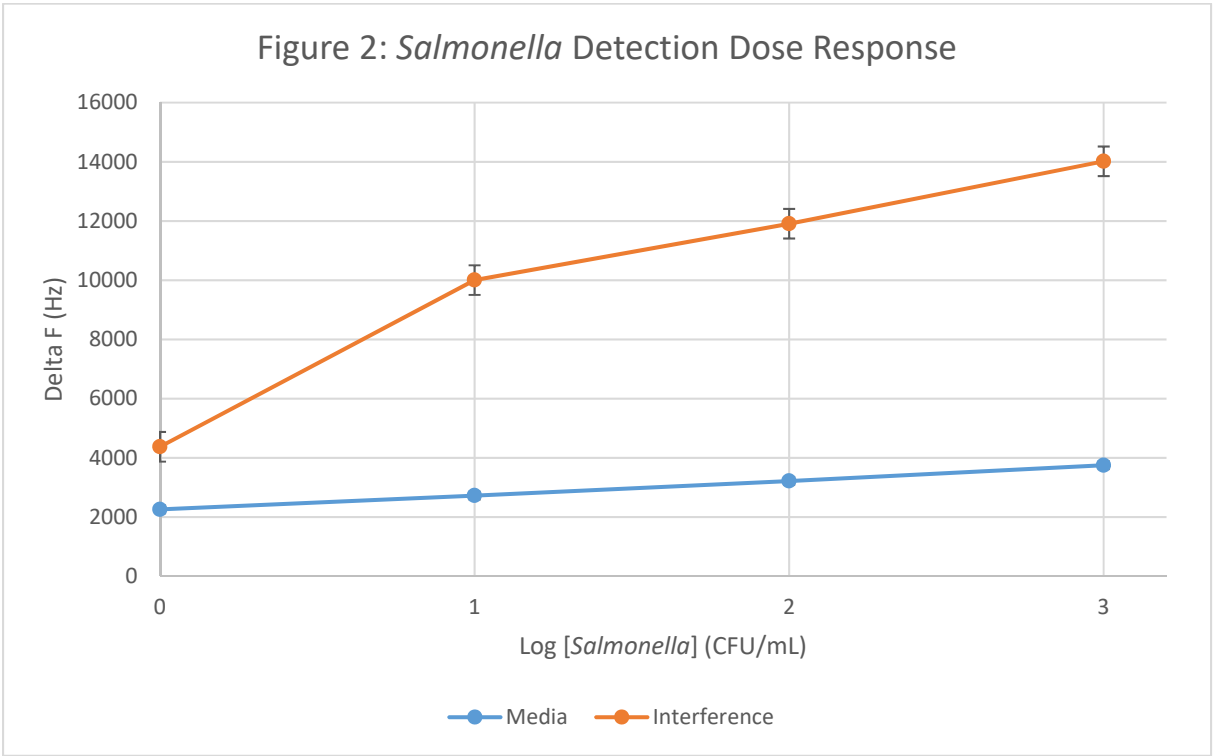
Figure 2: Salmonella Detection Dose Response
Delta F (Hz)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
1
2
3
Log [Salmonella] (CFU/mL)
Media
Interference

MAJOR HISTOCOMPATIBILITY COMPLEX AND GENETIC RESISTANCE TO INFECTIOUS BRONCHITIS VIRUS

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ABSTRACT

Infectious bronchitis virus (IBV) is an endemic disease of chickens responsible for considerable economic losses to the poultry industry worldwide. IBV replication leads to considerable viral genetic variability, and vaccine failures are commonly seen in the field. For this reason, new strategies to prevent infection and induce disease resistance must be investigated. Genetic resistance or susceptibility to infectious diseases have been largely associated with the avian major histocompatibility complex (MHC) genes. UC Davis has congenic chicken lines that share the same genetic background and vary solely in their MHC haplotype. Our goal was to determine the resistant congenic chicken lines available at UC Davis to use them as a tool for understanding the mechanisms of resistance to IBV. We analyzed and compared immunological responses and the effect of challenge in different congenic and inbred chicken lines.

INTRODUCTION

The avian MHC has been largely associated with genetic resistance to several infectious diseases (3, 5, 7, 8), including IBV (1, 2). UC Davis has congenic chicken lines that share the same genetic background and vary solely in their MHC haplotype (9). We challenged congenic chicken lines available at UC Davis with an IBV Massachusetts 41 (M41) strain to determine which lines are relatively resistant or susceptible to IBV infection. This was accomplished by comparing clinical signs, pathology and humoral responses after challenge. Our objective was to determine the relative resistance and susceptibility of the chicken lines to IBV and establish a model to understand immunity against IBV and the relationship between the MHC and innate immune responses.

MATERIALS AND METHODS

Five MHC B haplotype congenic lines (253/B18, 312/B24, 331/B2, 335/B19 and 336/BQ), one inbred line (003/B17), and one commercial line of broilers were used in this experiment. Twenty-five day-old chicks of each line were raised in isolated rooms, totalizing 175 animals. Sera was collected at 21 days of age to detect maternal antibodies against IBV by ELISA.

At 23 days of age, all birds were challenged with a M41 strain of IBV via oculonasal route using a median embryo infective dose (EID_{50}) of $5x10^7$ in a final volume of 200 µL. At two and six days post-infection (DPI), tears were collected from all chickens for viral load assessment by RT-qPCR (6). Respiratory signs were assessed and indexes were calculated based on the severity of respiratory disease (10). Five birds per group were euthanized at each time point, and tracheas were collected for histopathology and histomorphometry (tracheal epithelial thickness measurement). At 14 DPI, tears and sera were collected from all remaining birds for IgA and IgG measurement by ELISA.

Clinical signs, viral load, histomorphometry measurements, and antibody levels were analyzed individually and compared by one-way ANOVA followed by Tukey multiple comparisons test using GraphPad Prism software (GraphPad, La Jolla CA, USA). Statistical differences were considered at a significance level of $P<0.05$.

RESULTS

All chickens presented respiratory signs after challenge. However, mortality was not observed after infection. At two DPI, the MHC B congenic chicken line 335/B19 showed the lowest respiratory sign index and the milder tracheal inflammation. On the contrary, clinical signs were more severe in 077/B19 inbred chicken line. There were no statistical differences among groups regarding respiratory signs and histomorphometry measurements at six DPI. Lines 077/B19 and 253/B18 showed the lowest viral load at two DPI while broilers and 331/B2 showed the lowest viral load at six DPI, suggesting reduced viral shedding and consequently decreased viral replication due to early viral neutralization. Broilers presented the highest levels of serum IgG. IgA in tears was significantly higher in 331/B2 in comparison to the

other tested groups. All results are summarized in Table 1.

DISCUSSION

Even though lines 335/B19 and 077/B19 present the same MHC haplotype, the results obtained in both lines were divergent. The discrepancy is probably due to the fact that these lines do not share the same genetic background, thus other loci might be playing a role in genetic resistance against IBV in line 077/B19. Although there were no statistical differences between groups regarding clinical signs and tracheal inflammation at six DPI, there is a trend suggesting that line 331/B2 is more resistant than the others at 6 days after challenge. This trend can also be observed when considering the high levels of IgA detected in chickens from line 331/B2. As IBV preferred site of replication is locally at the upper respiratory tract, IgA levels are more likely related to protection against IBV than systemic IgG titers.

Relative resistance and susceptibility findings match what has been observed in previous experiments from our group (4) and what has been described in literature (1, 2), in which B2 and B19 present resistant and susceptible characteristics respectively. Susceptibility differences were observed early in infection, and are most likely related with differences in innate immune function. Our prospective studies will use the most resistant (331/B2) and the most susceptible (335/B19) congenic chicken lines as a model for understanding the mechanisms in which the innate immune system generates resistance to IBV in early infections. The goal is to use molecular tools and functional assays to unveil how the chicken lines respond to different strains of IBV and what are the cytokines and molecules involved in protection against IBV infection.

REFERENCES

1. Bacon, L. D., D. B. Hunter, H. M. Zhang, K. Brand, and R. Etches. Retrospective evidence that the MHC (B haplotype) of chickens influences genetic resistance to attenuated infectious bronchitis vaccine strains in chickens. Avian pathology : journal of the W.V.P.A 33:605-609. 2004.

2. Banat, G. R., S. Tkalcic, J. A. Dzielawa, M. W. Jackwood, M. D. Saggese, L. Yates, R. Kopulos, W. E. Briles, and E. W. Collisson. Association of the chicken MHC B haplotypes with resistance to avian coronavirus. Developmental and comparative immunology 39:430-437. 2013.

3. Briles, W. E., R. W. Briles, D. L. Pollock, and M. Pattison. Marek's disease resistance of B (MHC) heterozygotes in a cross of purebred Leghorn lines. Poultry science 61:205-211. 1982.

4. da Silva, A. P., R. Hauck, and R. A. Gallardo. Major histocompatibility complex effects on acquired immunity and genetic resistance to infectious bronchitis virus. In. 2017, manuscript in preparation.

5. Dawes, M. E., L. M. Griggs, E. W. Collisson, W. E. Briles, and Y. Drechsler. Dramatic differences in the response of macrophages from B2 and B19 MHC-defined haplotypes to interferon gamma and polyinosinic:polycytidylic acid stimulation. Poultry science 93:830-838. 2014.

6. de Quadros, V. L. Das Infektiöse Bronchitis Virus (IBV): Molekularbiologische Untersuchungen zur Diagnostik und zum Vorkommen sowie zur Pathogenität des Genotyps IBV QX in spezifisch pathogenfreien (SPF) Broilern. In: Institut für Geflügelkrankheiten. Freien Universität Berlin, Berlin. p 141. 2012.

7. Kaufman, J. The Avian MHC In: Avian Immunology, 2 ed. K. A. Schat, B. Kaspers and P. Kaiser, eds. Academic Press, Boston. pp 149-167. 2014.

8. Liu, W., M. M. Miller, and S. J. Lamont. Association of MHC class I and class II gene polymorphisms with vaccine or challenge response to Salmonella enteritidis in young chicks. Immunogenetics 54:582-590. 2002.

9. Pisenti, J. M., M. E. Delany, R. L. Taylor Jr, U. K. Abbott, H. Abplanalp, J. A. Arthur, M. R. Bakst, C. Baxter-Jones, J. J. Bitgood, F. A. Bradley, K. M. Cheng, R. R. Dietert, J. B. Dodgson, A. M. Donoghue, A. B. Emsley, R. J. Etches, R. R. Frahm, R. J. Gerrits, P. F. Goetinck, A. A. Grunder, D. E. Harry, S. J. Lamont, G. R. Martin, P. E. McGuire, G. P. Moberg, L. J. Pierro, C. O. Qualset, M. A. Qureshi, F. T. Shultz, and B. W. Wilson. Avian Genetics Resources at Risk: An assessment and proposal for conservation of genetic stocks in the USA and Canada. UC Davis Genetic Resources Conservation Program, Davis. 1999.

10. Toro, H., V. L. van Santen, L. Li, S. B. Lockaby, E. van Santen, and F. J. Hoerr. Epidemiological and experimental evidence for immunodeficiency affecting avian infectious bronchitis. Avian Pathology 35:455-464. 2006

Table 1. Respiratory signs, histomorphometry of tracheal epithelia, deciliation of tracheas, viral load in tears, IgG levels in sera and IgA levels in tears in MHC B congenic chicken lines at 2, 6 and 14 days post-infection (DPI).

Chicken lines	Respiratory signs 2 DPI	Respiratory signs 6 DPI	Histomorph-ometry 2 DPI	Histomorph-ometry 6 DPI*	Deciliation 2 DPI	Deciliation 6 DPI	Viral Load 2 DPI	Viral Load 6 DPI	IgG 14 DPI (S/P ratio)	IgA 14 DPI (OD 650 nm)
253/B18	1 3.33[a]	3 1.67	4 12.6[a]	1 156	6 0	9 3.33	2 5.34[bc]	2 6.69[a]	0.3 045[ab]	0. 1672[ab]
077/B19	4 1.18[b]	6 3.89	4 06.3[a]	1 065	8 0	9 3.33	2 4.38[c]	2 4.99[b]	0.4 174[ab]	0. 1839[ab]
Broilers	2 0.51[ab]	5 2.38	1 047[b]	1 180	5 3.33	7 3.33	2 6.41[ab]	2 1.77[c]	0.4 386[a]	0. 1192[b]
312/B24	1 8.06[a]	4 9.12	4 42.3[a]	8 63.5	8 0	8 6.67	2 6[ab]	2 5.57[ab]	0.2 439[ab]	0. 118[b]
331/B2**	**1 4.67[a]**	**3 1.67**	**3 39[a]**	**8 28.8**	**6 6.67**	**8 8.89**	**2 5.56[b]**	**2 4.96[b]**	**0.2 371[ab]**	**0. 2277[a]**
335/B19***	**9. 72[a]**	**4 0.35**	**3 31.6[a]**	**9 40.3**	**7 3.33**	**1 00**	**2 6.54[a]**	**2 5.5[b]**	**0.1 939[b]**	**0. 1009[b]**
336/BQ	1 3.04[a]	4 0.74	4 10.5[a]	1 146	1 00	1 00	2 6.33[ab]	2 5.53[ab]	0.2 028[ab]	0. 1704[ab]

* No statistical differences observed between groups

** Relatively resistant chicken line in comparison to other lines tested

*** Relatively susceptible chicken line in comparison to other lines tested

SEROPREVALENCE OF CHICKEN ASTROVIRUS GROUP B ASSOCIATED WITH "WHITE CHICK" DISEASE IN BROILER BREEDERS IN THE US AND CANADA

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ABSTRACT

Chicken astroviruses were first characterized by Baxendale and Mebastion (2004) from cases of runting stunting syndrome in the Netherlands. Since then, CAstVs have been detected in numerous countries and have been associated with several disease conditions, including visceral gout, nephritis, growth depression, and "white chick syndrome" (WCS). Within the US and Canada, an increasing number of reports concerning WCS have recently emerged. Two antigenically distinct groups of CAstV, designated A and B, have been identified based on the capsid protein (ORF2) sequences. Comparisons of the sequences have shown that both Groups A and B are currently each comprised of 3 subgroups (Ai, Aii, and Aiii, Bi, Bii, Biii). Determination of CAstV antibodies in serum samples provides an easy way to determine the prevalence of virus infections within and between various flocks. Earlier developed serological assays such as the indirect immunofluorescence (IF) tests show low levels of cross reactivity between groups A and B, are labor intensive, and time consuming. Therefore, an indirect enzyme-linked immunosorbent assay (ELISA) for the serological detection of Group B Chicken Astrovirus antibodies was developed. The test can be used as a screening tool and to assist in the diagnosis of Group B CAstV infections. Results show that the BioChek CAstV-gB ELISA detects antibodies to CAstV Bi, Bii, and Biii, but not to CAstV Group A. Testing of 459 sera from specific-pathogen-free (SPF) chickens showed greater than 99% specificity. Results from over 2,000 serum samples obtained from broiler breeders in the US and Canada suggest that broiler flocks with WCS and confirmed to be infected with CAstV were derived from hens sourced from pullets which were mostly negative for CAstV-gB antibodies based on the ELISA. After infection, CAstV-gB ELISA titers increased in affected hens relative to non-infected hens. Furthermore, broiler breeder flocks greater than 27 weeks of age in the US were predominately positive. In contrast, similar age flocks in Canada were predominately negative with positive flocks showing less than 50% of the birds in positive flocks testing positive. In conclusion, the BioChek CAstV-gB ELISA provides a convenient serological tool to diagnose Group B CAstV infections. In broiler breeders it can be used to test flocks prior to lay to identify seronegative flocks which may be at risk for an infection during lay. Currently, some companies which have experienced disease conditions associated with CAstV infection are moving litter from known positive to known negative flocks because there are no vaccines available. The assay can be used to confirm exposure post litter movement. In the future when vaccines are available, it can be used to assess the antibody response induced by vaccination. Lastly, the CAstV-gB ELISA can be used to demonstrate freedom from Group B CAstV infections in SPF flocks.

INFECTIOUS LARYNGOTRACHEITIS GENOMICS: WHAT'S CIRCULATING IN THE BACKYARD FLOCKS FROM THE UNITED STATES?

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INTRODUCTION

Phylogenetic and comparative genomic studies of infectious laryngotracheitis virus has mainly focused on the differences among live attenuated vaccines, vaccinal revertants and natural recombinant strains from Australia, the US, and China. This analysis suggests that most strains from the US have a close relationship with the vaccine viruses and are largely considered vaccinal revertants. In a search to identify wild type parental strains, the genomes of five ILTV isolates from backyard and commercial flocks in the US were sequenced using the MiSeq technology. Two isolates, S2.816 and 6.48.88 showed evolutionary relatedness to the vaccine strains SA2 and A20 licensed for use in Australia. Virulent strain 3.26.90 isolated from a backyard flock shared 99.9 % sequence identity with the genome of the commercial ILTV vaccine strain CEO TRVX as did 14.939, a commercial flock isolate from the mid-Atlantic region. Only one newly sequenced genome J2 partitioned outside the vaccine clades sharing 96.2% sequence identity with the genome of virulent isolate 1874C5. A diagnostic assay was developed to differentiate ILTV strains based on single nucleotide polymorphisms in the loci encoding ORFA and ORFB from 27 complete ILTV genomic sequences within GenBank. This assay will be useful to quickly identify non-vaccinal revertants in order to develop new vaccines against circulating field viruses. Even though modified-live ILTV vaccines TCO and CEO have been used for decades in the US, an extensive recombination study failed to detect any ILTV recombinants that were the progeny of these two licensed vaccines.

MATERIAL AND METHODS

Origin of the virus isolates. Tracheal swabs were collected from chickens experiencing clinical signs of ILT. Isolate 14.939 came from a broiler on a commercial farm for the mid-Atlantic region of the US in 2014. All other isolates were collected for backyard flocks. Isolate 6.48.88 and 3.26.90 came from backyard flocks collected in 1988 and 1990, respectively. S28.16 was from a backyard Peafowl (circa 2002) and J2 was isolated from a fighting cock from the southwestern part of the US (circa 2009). Table 1 lists all the ILTV strains used in this comparative genomic study. Virus isolation was done as previously published (1). Viruses were first propagated in primary chicken kidney cells (CK) for five to six days. Subsequent passaged stocks were treated as cell-free preparations. Titers were determined using the TCID50 protocol (2). In order to isolate viral DNA, CK cells were infected with virus isolates using multiplicity of infection of 0.01-0.0001, depending on the growth characteristics of the virus. Nucleocapsid DNA was isolated using the micrococcal nuclease method (3).

Genome sequencing, assembly and alignments. The nucleotide sequences of the five ILTV genomes were determined using the Illumina MiSeq platform. De novo assembly was performed using mira v3.4.0.1 (4). Assembly quality control and finishing were performed using Gap4 from the Staden package (5). Genome annotation was performed using an in-house herpesvirus annotation pipeline. Multiple sequence alignments of the complete genome sequences were performed using the online program MAFFT (6). SNPs and INDELS were identified by comparisons with all 22 ILTV complete genomic sequences in GenBank. The amino acids corresponding to SNPs were identified using MEGA 4.0 phylogenetic analysis (Maximum Parsimony method) (7).

Recombination analysis. Recombination between ILTV strains was investigated using the bootstrap analysis program Simplot v3.5.1 in order to detect possible break points on selective sequences representatives of different clades (8). Additionally, statistical analysis of the recombination events was done using the Recombination Detection Programs (v 4.46) suite of recombination programs (RDP, GENECONS, Bootscan, MaxChi, Chimaera and 3Seq with the circular ILTV genomes (9).

Single locus amplicon. In order to identify genomic loci suitable for single-locus genotyping ("molecular classifiers"), complete genome sequences

from 16 published ILTV genomes and the newly sequenced genomes were aligned using MAFFT (after removal of redundant 3' TRS region). Each of the 21 genomes was assigned to one of six predetermined classes based on known genotyping data. Candidate loci were then chosen and ranked based on genomic length (with smaller loci and therefore smaller amplicons preferred). Loci containing Iltovirus-specific genes were prioritized and investigated further to determine their suitability based on amplification conditions and likelihood that a real time PCR assay could be successfully developed with a minimum number of fluorescent probes.

RESULTS AND DISCUSSION

Tracheal swabs were collected from chickens experiencing clinical signs of ILT. Table 1 lists all the ILTV strains used in this comparative genomic study. Nucleotide sequences obtained by MiSeq sequencing were assembled de novo. The five genomes assembled as 154,001 (S2.816), 154,022 (6.48.88), 153,711 (J2), 153,655 (3.26.90) and 153,629 (14.939) base pairs (bp) in length. The phylogenetic relationships (Fig. 2) among the S2.816, 6.48.88, J2, 3.26.90 and 14.939 genomes with those from Europe, Australia and China reveal five clades. Clusters are labeled according to the nine genotypes as defined by a previous study (10). The US virulent strains 6.48.88 and S2.816 and the Australian vaccine strains SA2 and A20 form the group VI and VIII cluster, part of a larger branch that included virulent strains from both China (WG) and Australia (CSW-1 and VI-99). The majority of the strains including 3.26.90 and the Chinese and European vaccine strains K317 and Serva, respectively, clustered within the CEO clade (group IV), as does two CEO revertant strains, LJS09 (China) and ACC78 (Australia). Strain 14.939 and 63140 formed their own cluster, the virulent CEO-like viruses (group V). None of the newly sequenced strains partitioned within the TCO clade. However, US strains J2 and 1874C5 formed a single cluster related to both the TCO clade and a lineage branching immediately with virulent Australian strain CL9 and other virulent Australasian strains CSW-1 and VI-99.

Historically, a collection of informative regions (e.g. gB, gC, gM, gG, TK, ICP4, ICP18.5) have been used in various combinations to classify ILTV isolates into genotypes. The broad conclusion from these genotyping studies involving samples collected from Europe, Australia and the US is that most of the field isolates have RFLP patterns similar to those from vaccines strains. Although useful these multiple amplicon assays involved sequencing thousands of base pairs. To improve on this, 36 regions in the genome (927-1993 bp in length) were identified that contained at least 10 informative SNPs that could group the US strains into five classes. One locus encoding ORF A and ORF B was chosen. Five SNPs within this 1,751 bp region allowed for the differentiation of the 27 Strains of ILTV into five classes (Table 2).

In addition to vaccinal revertants related to the CEO lineage (11) and to a limited degree the TCO lineage (stain 18648), there are reports from Australia and China that virulent ILTV "natural" recombinants comprised solely or partially from genomic regions of vaccine strains are circulating in the field (12, 13). To investigate whether US field viruses isolated from backyard flocks were recombinants of two vaccine parents, Bootscan analysis was used to identify break points in the genomes of virulent and avirulent strains which were statistically analysed using the RDP program. Even though modified-live ILTV vaccines TCO and CEO have been used for decades in the US an extensive recombination study failed to detect any ILTV recombinants that were the progeny of these two licensed vaccines.

The geographic separation of the ILTV strains is still a mystery. In this report four virulent isolates (6.48.88, S3.816, 3.26.90 and 14.939) from different regions of the US showed evolutionary relatedness to different vaccine strains licensed for use on two continents (US and Australia). Based on phylogenetic relatedness US strains S2.817 isolated from Peafowl and the backyard flock isolate 6.48.88 are derivatives of the AU vaccine strain SA2. Whether these virulent US strains are the parental strains of SA2 or its vaccinal revertants is not known.

CONCLUSION

The phylogenetic tree suggest that half of virulent US strains sequenced so far are vaccinal revertants. The majority (i.e. 3.26.90, 63140 and 14.939) are related to CEO. Only one US strain (81658) is likely to be a vaccinal revertant of TCO. Similarly, virulent strains ACC78 and LJS09 from Australia and China respectfully, also appear to be CEO-revertants. Virulent viruses 1874C5 and J2 are likely wild type ILTV strains circulating in the US since they are distantly related to any vaccine strain and form their own clade, group VI. We also found no evidence of vaccinal recombination to form new virulent US strains using a battery of recombination prediction computer programs.

REFERENCES

1. Garcia, M. and S. Riblet, Characterization of infectious laryngotracheitis virus isolates:

demonstration of virus subpopulations with an vaccine preparations. Avian Dis., 2001. 45: p. 558-66.

2. Reed, L.J. and H. Muench, A simple method of estimating fifty percent endpoints. Am. J. Hygiene, 1938. 27,: p. 493-497.

3. Volkening, J.D. and S.J. Spatz, Purification of DNA from the cell-associated herpesvirus Marek's disease virus for 454 pyrosequencing using micrococcal nuclease digestion and polyethylene glycol precipitation. J Virol Methods, 2009. 157(1): p. 55-61.

4. Chevreux, B., T. Wetter, and S. Suhai, Genome Sequence Assembly using Trace Signal and Additional Sequence information. Computer Science and Biology GCB, 1999. 1: p. 45-56.

5. Bonfield, J.K., K. Smith, and R. Staden, A new DNA sequence assembly program. Nucleic Acids Res, 1995. 23(24): p. 4992-9.

6. Katoh, K., et al., MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res, 2005. 33(2): p. 511-8.

7. Kumar, S., K. Tamura, and M. Nei, MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform, 2004. 5(2): p. 150-63.

8. Martin, D.P., et al., A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. AIDS Res Hum Retroviruses, 2005. 21(1): p. 98-102.

9. Martin, D.P., et al., RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol, 2015. 1(1): p. vev003.

10. Oldoni, I. and M. Garcia, Characterization of infectious laryngotracheitis virus isolates from the US by polymerase chain reaction and restriction fragment length polymorphism of multiple genome regions. Avian Pathol, 2007. 36(2): p. 167-76.

11. Guy, J.S., H.J. Barnes, and L. Smith, Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis, 1991. 35(2): p. 348-55.

12. Blacker, H.P., et al., Epidemiology of recent outbreaks of infectious laryngotracheitis in poultry in Australia. Aust Vet J, 2011. 89: p. 89-94.

13. Zhao, Y., C. Kong, and Y. Wang, Multiple Comparison Analysis of Two New Genomic Sequences of ILTV Strains from China with Other Strains from Different Geographic Regions. PLoS One, 2015. 10(7): p. e0132747.

Table 1. Strains used in comparison genomic study.

US Isolates & Vaccines	Origin	Year	Genome	Accession #
S2.816	Pea Fowl[d]	2002	154,001	Not
6.48.88	Backyard Flock[d]	1988	154,022	Not
J2	Game Chickens[d]	2008	153,711	Not
1874C5	Broiler	2004	150,689[e]	JN542533
14.939	Broiler	2014	153,629	Not
63140	Broiler	2006	153,633	JN542536
3.26.90	Backyard Flock[d]	1990	153,655	Not
CEO_TRVX[a]	Vaccine Trachivax	1983	153,647	JN580313
CEO_LP[c]	CEO low passage	1990	153,641	JN580317
CEO_HP[c]	CEO high passage	1990	153,647	JN580316
LT-Blen	CEO Vaccine	1975	153,639[e]	JQ083493
Laryngo-Vac	CEO Vaccine	1975	153,640[e]	JQ083494
SERVA	European CEO		153,645[e]	HQ630064
TCO-IVAX[b]	Vaccine IVAX	1983	155,465	JN580312
TCO_LP[c]	TCO low passage	1991	155,465	JN580315
TCO_HP[c]	TCO high passage	1991	150,335	JN580314
USDA	Challenge strain	1960	151,767[e]	JN542534
81658	Broiler Breeder	2010	150,335	JN542535
Australian Isolates &				
CSW-1	Layer	1970	151,671	JX646899
V1-99	Layer	1999	153,630	JX646898
CL9	Broilers	2008	152,650[e]	JN804827
ACC78	Broilers/layers	2008	153,645[e]	JN804826
A20	Vaccine	1966	153,985[e]	JN596963
SA2	Vaccine	1983	153,982[e]	JN596962
Chinese Isolates & Vacc				
LJS09	Layer	2009	153,200[f]	JX458822
K317	Layer		153,638[f]	JX458824
WG	Vaccine	1950	153,504[f]	JX458823

[a]Chicken embryo origin (CEO) vaccine; [b] Tissue culture origin (TCO) vaccine; [c] High and low chicken passages of the CEO and TCO vaccines (Guy et al., 1991); [d] Backyard flock strains, otherwise strains are either of commercial poultry origin or vaccines; [e]Lengths corrected for missing 3' TRS sequences; [f]Residue (T) removed from 3' end.

Figure 1. Phylogenetic tree of 27 ILTV strains. A multiple alignment of full-length genome sequences (TRS removed) was generated using MAFFT v7.205 in fast global alignment mode. Gap positions were stripped and the phylogenetic tree was generated using PhyML v20130805 with SH-like branch support. Branches with support values < 0.6 were collapsed. One hundred bootstrap replicates were used to access the significance of the tree topology. The genomes of the newly sequenced isolates are shown in boxes. Clusters are labeled according to the 9 genotypes as defined by Oldoni & Garcia [10].

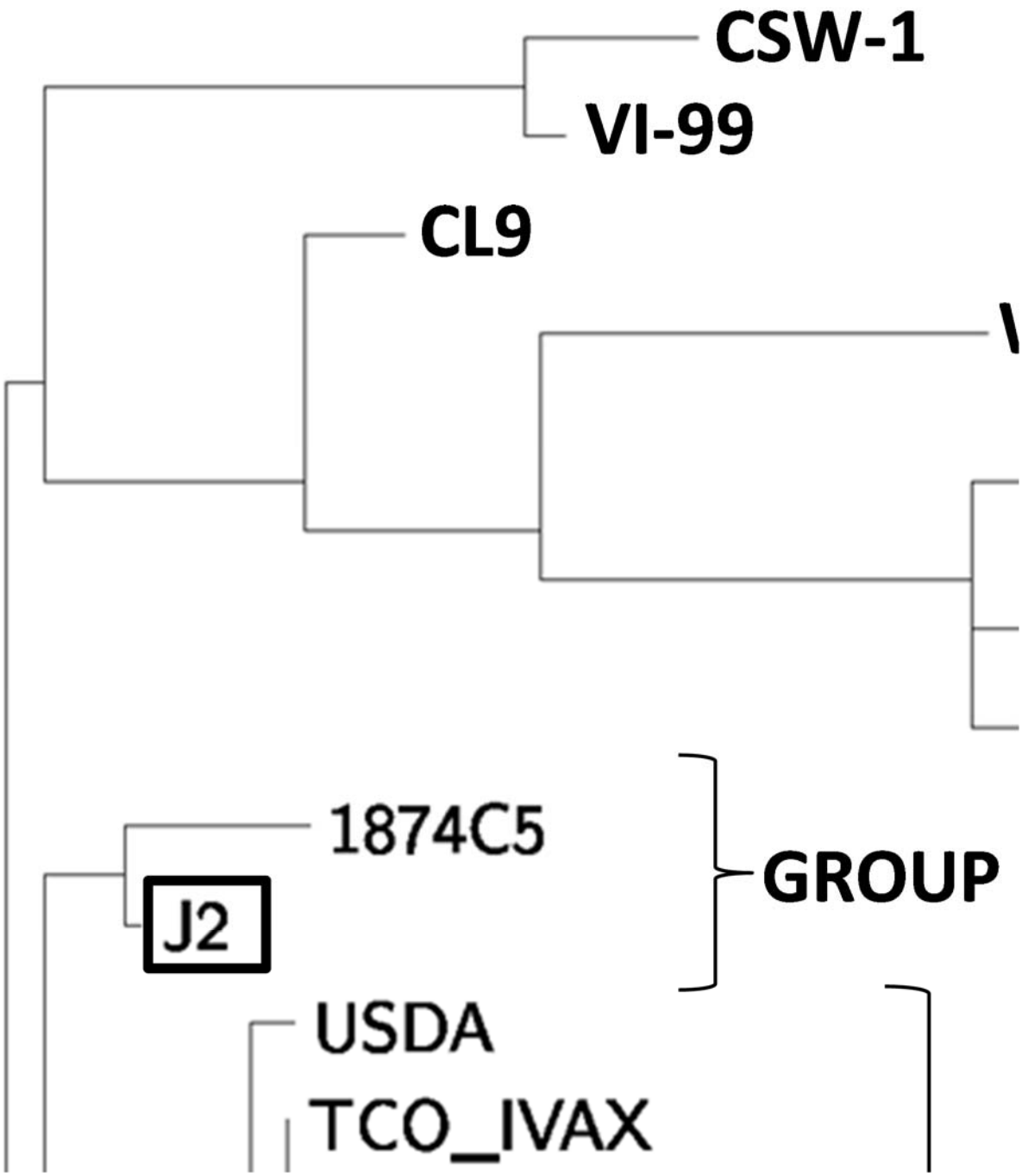

Table 2. Differentiating SNPs in the single locus assay. Numbers on top of the columns are the positions of the informative SNPs within the amplicon.

In silico genotyping of US strains of ILTV by multigene and single locus sequencing							
	Multiple genes	Single Locus SNPs					Single Locus
Strains	Groups	131	929	1277	1312	1614	Groups
USDA	I	C	C	A	G	AG	I,II,III
TCO-IVAX	II	C	C	A	G	AG	
TCO_LP	II	C	C	A	G	AG	
TCO_HP	II	C	C	A	G	AG	
81658	III	C	C	A	G	AG	
CEO_TRVX	IV	C	T	A	G	AG	IV
CEO_LP	IV	C	T	A	G	AG	
CEO_HP	IV	C	T	A	G	AG	
LT-Blen	IV	C	T	A	G	AG	
Laryngo-VAX	IV	C	T	A	G	AG	
3.26.90	IV	C	T	A	G	AG	
63140	V	C	T	C	G	AG	V
14.939	V	C	T	C	G	AG	
1874C5	VI	T	T	A	T	AG	VI
J2	VI	T	T	A	T	AG	
S2.816	VII	C	T	A	T	GA	VII,VIII
6.48.88	VIII	C	T	A	T	GA	
In silico genotyping of AU and CN strains of ILTV by multigene and single locus sequencing							
Acc78	IV	C	T	A	G	AG	IV
CL9	IV	C	T	A	G	AG	
Serva	IV	C	T	A	G	AG	
LJS09	IV	C	T	A	G	AG	
K317	IV	C	T	A	G	AG	
WG	IV	C	T	A	G	AG	
CSW-1	VII	T	T	A	T	AG	VI
VI99	VII	T	T	A	T	AG	
A20	VII	C	T	A	T	GA	VII
SA2	VII	C	T	A	T	GA	

HOW FAST WAS HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS SPREADING WITHIN TURKEY FLOCKS DURING THE 2015 H5N2 EPIDEMIC IN THE UNITED STATES?

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SUMMARY

Daily flock mortality data from the 2015 H5N2 highly pathogenic avian influenza (HPAI) virus epidemic in turkeys in Minnesota were used to estimate within-flock transmission rate parameters. First, daily mortality data, together with the total number of birds placed, were used to estimate the number of newly infected (C), susceptible (S), infectious (I), and the total number (N) of turkeys at different time points within each flock. Then, Generalized Linear Model (GLM)-based epidemic modeling techniques were used to estimate the adequate contact rate (transmission rate parameter β), and subsequently, the basic reproduction number R_0. It was found that every infectious turkey would potentially infect 2.24 turkeys per-day (95% confidence interval (C.I.): 1.66 – 3.01). When considered over its entire infectious period of four days, on average, an infectious turkey would infect up to 8.96 (95% C.I.: 6.64 – 12.04) additional turkeys, which gives the within-flock R_0. Epidemiologically, $R_0>1$ implies that a major within-flock epidemic would be expected in an exposed flock as indicated by our estimates. This study emphasizes the importance of accurate recording of daily mortality data by producers and emergency responders in the field. Analysis of outbreak data is critical for understanding within-flock transmission dynamics of avian influenza. Model parameter estimates from this analysis will be used in HPAI emergency response and preparedness planning. A full-length article will be published in a (yet-to-be determined) refereed journal.

INTRODUCTION

Between December 2014 and June 2015, outbreaks of highly pathogenic avian influenza (HPAI) viruses caused substantial economic losses in the poultry industry in the United States. Outbreaks affected 232 domestic flocks in 15 states (with 160 commercial turkey operations as the majority) resulting in culling of approximately 49.6 million birds (3). The total impact on the U.S. economy was estimated to be close to USD 3.3 billion (6). Control of future HPAI outbreaks will require the design and evaluation of effective surveillance and product movement biosecurity protocols. The design and evaluation of these protocols will in part be informed by the knowledge gained from analyses of outbreak data. This includes a better understanding of within-flock HPAI transmission using simulation models with reliable parameter estimates. For example, HPAI within-flock spread simulation models are used in Secure Poultry Supply Plan risk assessment studies to evaluate active and passive surveillance protocols, as well as for predicting a flock's level of infectivity to neighboring flocks during the epidemic. Within-flock transmission dynamics impact risk estimates made from quantitative models used in risk assessment, therefore the primary objective of this study was to estimate the within-flock adequate contact rate or transmission rate parameter β, which is the mean number of birds infected by one infectious bird per unit time (in a naïve population). Thus, β determines the disease spread rate and its corresponding basic reproduction number R_0 (R_0 = β x infectious period) (4).

MATERIALS AND METHODS

Daily flock mortality data are regularly collected as part of routine activities in the poultry industry. Daily mortality data collected during the outbreak were used here to determine the HPAI virus spread rate (β) within turkey flocks infected with H5N2 HPAI virus. Ideally, β estimation requires data on the number of C, S, I, and N birds at different time points.

Through back-calculation (2), we estimated these four variables from available daily flock mortality data, assuming fixed latent and infectious periods of one and four days respectively. The latent and infectious periods were estimated based on data from inoculation studies in turkeys and rounding to get integer values (personal communication, Dr Erica Spackman).

Once C, S, I and N were obtained, β and its 95% confidence interval (C.I.) were estimated using Generalized Estimating Equations (GEE) using the GENMOD procedure in SAS (SAS Institute Inc., Cary NC), with an exchangeable correlation structure and flock as the repeated subject (1). The confidence intervals were based on the empirical estimates. R_0 was then calculated as the product of the estimated β and the set (deterministic) infectious period. Details of the modeling approach can be found in (2).

RESULTS

Using the GEE approach with the set latent and infectious periods, the estimated β was 2.24 (95% C.I.: 1.66 – 3.01) birds per infectious bird per-day. The basic reproduction number R_0 was estimated to be 8.96 (95% C.I.: 6.64 – 12.04).

DISCUSSION

The magnitude of the transmission rate parameter β may influence the rate of HPAI virus spread and the rate of accumulation of HPAI disease mortality at various times post exposure of a turkey flock. The current analysis is the first to estimate the within-flock transmission parameter β from U.S. outbreak mortality data in turkeys. The U.S. outbreak data based estimates would be more relevant to inform outbreak control strategies relative to estimates from experimental data or from outbreaks in other countries. The transmission parameter β can be estimated from laboratory inoculation studies in poultry. However, extrapolations from experimental studies to commercial flocks may have a greater uncertainty because this parameter would depend on the bird species, production practices in a country, and the HPAI strain. Thus, whenever field data are available, efforts should be made to estimate country-specific transmission rate parameters. The availability of daily mortality data for model parameter development representative of management practices in the U.S. commercial poultry industry is critically important as shown in this study. Egg production data has been used in similar efforts (5). A key limitation of this study's approach is assuming deterministic latent and infectious periods (based on experimental data) in the back calculation procedure and not considering between-bird variation in these disease state durations. In addition, the current back calculation procedures also require using integer valued latent and infectious periods.

We conclude that back-calculation is a computationally efficient method that uses accepted GLM-based procedures to obtain reasonable estimates for β, which is a key parameter in a number of modeling analyses used for decision support during an emergency response and for the evaluation of active surveillance protocols during emergency preparedness planning. We emphasize that developing multiple methods to estimate β may improve the accuracy of within-flock HPAI spread model predictions. Timely access to outbreak data is critical for future analyses. Such efforts are made possible through joint industry, government and academic partnerships.

REFERENCES

1. Becker, N. G. Analysis of infectious disease data. Chapman and Hall, London. 1989.

2. Bos, M. E. H., M. Nielen, G. Koch, A. Bouma, M. C. M. de Jong, and A. Stegeman. Back-calculation method shows that within-flock transmission of highly pathogenic avian influenza (H7N7) virus in the Netherlands is not influenced by housing risk factors. Preventive Veterinary Medicine 88:278-285. 2009.

3. Dargatz, D., A. Beam, S. Wainwright, and B. McCluskey. Case Series of Turkey Farms from the H5N2 Highly Pathogenic Avian Influenza Outbreak in the United States During 2015. Avian Diseases 60:467-472. 2016.

4. Diekmann, O., J. A. P. Heesterbeek, and J. A. J. Metz. On the definition and the computation of the basic reproduction ratio R0 in models for infectious-diseases in heterogeneous populations. Journal of Mathematical Biology 28:365-382. 1990.

5. Gonzales, J. L., A. R. Elbers, J. A. van der Goot, D. Bontje, G. Koch, J. J. de Wit, and J. A. Stegeman. Using egg production data to quantify within-flock transmission of low pathogenic avian influenza virus in commercial layer chickens. Prev Vet Med 107:253-259. 2012.

6. Greene, J. L. Update on the Highly-Pathogenic Avian Influenza Outbreak of 2014–2015. Congressional Research Service. Online: https://fas.org/sgp/crs/misc/R44114.pdf. 2015.

OUTBREAK OF INCLUSION BODY HEPATITIS IN COMMERCIAL CALIFORNIA BROILERS

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SUMMARY

In 2016, there were 86 cases of acute inclusion body hepatitis diagnosed at the California Animal Health and Food Safety (CAHFS), Turlock Laboratory. Cases originated from commercial broilers flocks submitted from different premises owned by a single company. Affected broilers were between 12-27 days of age. Degenerative lesions were identified primarily in the livers, pancreas and kidneys with basophilic intranuclear inclusions identified in affected organs. Based on PCR and sequencing, the adenovirus isolated from livers was classified as a fowl adenovirus group E serotype 7.

INTRODUCTION

IBH in broilers was initially reported in North America in 1963 with rapid expansion to most poultry producing regions worldwide (2). Acute IBH typically affects chickens between three to seven weeks of age. Transmission occurs by both horizontal and vertical infection. Acute IBH results in a sudden onset of mortality and morbidity which peaks after three to four days post infection. Mortality can increase to 10% and a few cases have reported mortality rates as high as 30%. Mortality typically returns to normal after five to 10 days. Macroscopically the disease is characterized by pale friable, mottled livers and there may be petechial and ecchymotic hepatic hemorrhages (3). Enlarged mottled kidneys, icterus of skin, pale bone marrow and bursal atrophy have also been described. Histologically, there is acute hepatic necrosis with infiltration of basophilic intranuclear inclusions within hepatocytes (3).

Acute inclusion body hepatitis (IBH) in broilers is attributed to infection with double stranded DNA fowl adenoviruses (FAdV) from the genus aviadenovirus. FAdV are classified into five species (A-E) based primarily on molecular characteristics (6). FAdV within each species can be further subdivided into serotypes (1). Avian adenoviruses are ubiquitous in chickens and there is variability in virulence of strains. Traditionally FAdV strains have frequently been reported as opportunistic pathogens in chickens that are immune compromised by co-infection with either chicken infectious anemia or infectious bursal disease. In the past decade, there has been an emergence of virulent FAdV strains that act as primary pathogens in broilers in the absence of impaired immunity (5). Aviadenoviruses are resistant to infection and are highly contagious. Control of pathogenic strains in endemic areas is mainly achieved by the use of inactivated or live vaccines in breeders in order to confer maternal immunity in progeny (4). This case report describes an outbreak of IBH in commercial broilers attributable to FAdV group E serotype 7.

MATERIALS AND METHODS

A total of 86 necropsy cases of IBH were submitted to the CAHFS Turlock lab in 2016. Cases were submitted from multiple premises from commercial broiler flocks originating from one company. Affected broilers could be traced back to a specific broiler breeder source. Broilers submitted were typically between two to four weeks of age. A combination of live and dead broilers were submitted for diagnostic evaluation from flocks experiencing increased mortality between 5-10%. Chickens submitted were necropsied, and tissue sections were processed for histopathology using hematoxylin and eosin staining. Virus isolation using avian cell culture inoculation and electron microscopy was performed on liver tissue pools obtained from necropsy cases (CAHFS, Davis) and five isolates were subsequently selected for IBH PCR to detect adenovirus hexon gene and sequence analysis (The Poultry Diagnostic and Research Center, The University of Georgia, Athens). ELISA serology was performed for infectious bronchitis, Newcastle disease, *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), avian influenza (AI), avian reovirus and infectious bursal disease (IBD). Avian influenza RT-PCR was performed on oropharyngeal swabs from all cases. The organs selected for aerobic culture (5% Sheep's blood and MacConkey's agar) and additional diagnostic testing performed, such as IBD RT-PCR was not standard on the 86 necropsy submissions.

RESULTS AND DISCUSSION

Live chickens submitted were lethargic, with ruffled feathers and green diarrhea. At necropsy, the most consistent lesion was enlarged, mottled, livers with tan discoloration. There were petechial or ecchymotic hemorrhages on the livers of affected birds. Kidneys were pale enlarged and mottled in some of the affected birds. In a few chickens there was icterus of the skin and adipose tissue. Mild hydropericardium and diffuse pale foci were also reported throughout the pancreas in some birds. Microscopically, there was extensive hepatic degeneration with basophilic intranuclear inclusion bodies invading some hepatocytes. Moderate periportal mononuclear hepatitis was identified in some liver sections. There were multifocal areas of pancreatic acinar cell necrosis with intranuclear inclusions identified in the necrotic zones. In kidneys, renal tubular dilation and multifocal hyaline casts within the tubules was identified. Aplastic anemia, and mild hydropericardium were identified in a few submissions. In most of the IBH cases, there was no microbial growth from most of the IBH livers cultured.

All five of the adenovirus cell culture samples submitted for genotyping were positive for adenovirus by PCR. The nucleotide sequences of the five adenovirus hexon products were 99.1% similar to FADV group E serotype 7. All 86 chickens were negative for avian influenza by RT-PCR and serology. Serology was negative for MG, MS, ND, reovirus and infectious bronchitis. Lymphocyte depletion in the bursas was identified in 38.3% (33/86) of cases of IBH with 11/33 (33.3%) of these cases were confirmed as positive for infectious bursal disease by RT-PCR. In the other 22 cases of bursal atrophy, IBD PCR was not performed. From 2010-2015, the number of IBH cases diagnosed throughout the four CAHFS lab system ranged between 0-4 (Table 1). During this 2016 outbreak, 86 cases of IBH was diagnosed (Table 1).

Ranch managers typically reported a return to normal flock mortality after seven to 10 days in the absence of any treatment. The source of infection was traced back to a single breeder source. Infection is thought to have occurred vertically from breeders with subsequent horizontal transmission within affected flocks. In most of the case submissions, an immune compromised status characterized by lymphocyte depletion in the bursas was not a prerequisite for clinical disease. The exact role of simultaneous or previous infection with infectious bursal disease in the incidence and severity of IBH was not fully elucidated. The outbreak of IBH was managed by implementation of a killed autogenous vaccination program administered to broiler breeders at 11 and 18 weeks of age. Implementation of vaccination was successful in conferring maternal immunity to broiler progeny and significantly reduced the incidence of IBH cases.

ACKNOWLEDEMENT

Adenovirus sequencing and phylogenetics was done at Dr Holly Sellers Lab, The Poultry Diagnostic and Research Center, Department of Population Health, The University of Georgia, Athens.

REFRENCES

1. Calnek, B. W., and B. S. Cowen. Adenoviruses of Chickens: Serologic Groups. 19:91-103. 1975.

2. Helmbolt, C., and M. N. Frazier. Avian Hepatic Inclusion Bodies of Unknown Significance. Avian Dis. 7:446-450. 1963.

3. Howell, J., D. W. MacDonald, and R. G. Christian. Inclusion Body Hepatitis in Chickens. Can. Vet. J. 11:99-101. 1970.

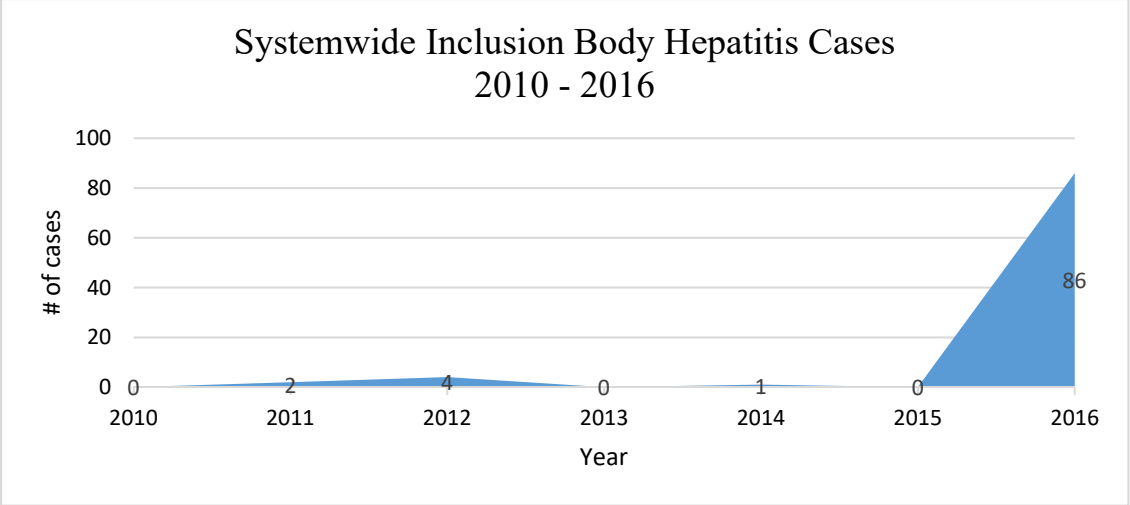
4. Saifuddin, M., and C. R. Wilks. Reproduction of inclusion body hepatitis in conventionally raised chickens inoculated with a New Zealand isolate of avian adenovirus. N. Z. Vet. J. 38:62-65. 1990.

5. Sumantha, G., R. Goodhope, D. Ojkic and P. Willson. Inclusion Body Hepatitis as a Primary Disease in Broilers in Saskatchewan, Canada. Avian Dis. 50:550-555. 2006.

6. Zsák L., and J. Kisary. Grouping of fowl adenoviruses based upon the restriction patterns of DNA generated by BamHI and HindIII. Intervirology. 22(2):110-4. 1984.

(A complete manuscript will be submitted to *Avian Diseases* journal.)

Table 1. Number of Cases of Inclusion Body Hepatitis Diagnosed at the California Animal Health and Food System Laboratory between 2010-2016.



PREVENTING AVIAN INFLUENZA OUTBREAKS: BACK TO THE BASICS

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SUMMARY

While it is always good to keep pace with the latest scientific and technological advances, more often than not, preventing outbreaks of avian influenza and other catastrophic poultry diseases requires only simple, "common-sense" methods. However, these methods must be clear, concise, practical, science-based, and adapted to each specific target audience. There is undoubtedly a large volume of information on biosecurity available in print and online through various web-based sources. Biosecurity guidelines may look good on paper but that is exactly what they are—guidelines. Biosecurity is definitely not a "one size fits all". It is therefore important for poultry growers or integrators to develop biosecurity programs that fit their specific needs and challenges.

In 2008, the United Nations Food and Agriculture Organization (FAO) prepared a comprehensive paper on biosecurity for highly pathogenic avian influenza (1). The paper discusses the basic principles of biosecurity within the overall framework of disease control and species- and sector-specific issues, and stresses the importance of situating biosecurity in appropriate economic and cultural settings. It also stresses the important role of communication.

Based on their starting definition of biosecurity as "implementation of practices that create barriers in order to reduce the risk of the introduction and spread of disease agents", the FAO paper stresses that people are key to correct implementation but that this must be formulated in terms of measures that are hard to avoid and easy to comply with.

The authors cite the three principal elements of biosecurity:

1) Segregation – creating and maintaining barriers to limit the potential opportunities for infected animals and contaminated materials to enter an uninfected site.

2) Cleaning - thoroughly cleaning materials (e.g. vehicles, equipment) that have to enter (or leave) a site to remove visible dirt and most of the virus that is contaminating the materials.

3) Disinfection - to inactivate any virus that is present on materials that have already been thoroughly cleaned.

The FAO paper also emphasizes that any biosecurity measure must be practical and proportionate to the risk for which it was developed. In addition, the paper points out that the practical design of biosecurity measures should be grounded firmly in three key considerations:

- Biosecurity recommendations should be developed for all component parts of the domestic poultry and captive bird sector, including intermediaries and service providers.
- In most locations, the emphasis should be on preventive biosecurity to decrease the risk of infection (bioexclusion), although biocontainment remains important.
- Those who will implement biosecurity measures should be involved in their design to ensure that they are feasible and sustainable.

According to FAO, this latter consideration touches the core of what biosecurity is all about, i.e., stakeholder "buy-in", without which any attempt to achieve effective and sustainable disease prevention and control will fail.

The United States is one of the most modern and efficient poultry producing countries in the world with strict biosecurity measures such as those recommended by FAO in place in the majority of poultry operations, particularly in commercial poultry. However, the country experienced a devastating outbreak of highly pathogenic avian influenza (HPAI) in 2014-2015. This outbreak is the largest animal health emergency in the history of the United States, according to Dr. Kevin Shea, USDA-APHIS Administrator. The outbreak wiped out 10% of the entire layer inventory of the U.S. and reduced the national turkey inventory by 7.5%. The USDA destroyed 42 million layers and pullets and 7.5 million turkeys, and paid $190 million in indemnity. In his testimony at a U.S. Senate Hearing on July 7, 2015, Dr. John Clifford, USDA-APHIS Deputy Administrator pointed out that lateral spread of HPAI occurred when biosecurity measures that are sufficient in ordinary times were not sufficient in the face of such a large amount of virus in the environment (4). In his analysis of this unprecedented outbreak, Dr. Simon Shane, a consultant poultry veterinarian and biosecurity expert, concluded that "the U.S. poultry industry operated according to a standard of structural

and operational biosecurity incapable of protecting flocks from the introduction of a highly pathogenic virus. The injudicious concentration of large complexes with up to five million hens in close proximity, based on financial expediency and least-cost production, was contrary to principles of sound conceptual biosecurity and exacerbated the losses sustained following the introduction of HPAI into a county" (2).

According to a 2015 epidemiological study by USDA-APHIS on the 2014-2015 HPAI outbreak, "Although APHIS cannot…. point to a single statistically significant pathway for the current spread of HPAI, a likely cause of some virus transmission is insufficient application of recommended biosecurity practices (3). For example, APHIS has observed sharing of equipment between an infected and noninfected farm, employees moving between infected and noninfected farms, lack of cleaning and disinfection of vehicles moving between farms, and reports of rodents or small wild birds inside poultry houses." These findings highlight the need to review and reinforce "common sense" biosecurity practices. Education using practical, science-based communication of biosecurity measures is an essential and critical component of renewed programs to prevent future outbreaks of HPAI. For example, short biosecurity videos for commercial poultry growers, technical service personnel, and backyard flock owners showing how avian influenza virus spreads and practical, science-based biosecurity procedures to prevent the spread of AI were developed by the University of Maryland poultry Extension team through a USDA-NIFA Smith-Lever Special Needs Program grant. One of these videos will be presented at this meeting.

REFERENCES

1. Food and Agriculture Organization of the United Nations (2008; reprinted 2009). Biosecurity for highly pathogenic avian influenza: issues and options. Available at: http://www.fao.org/3/a-i0359e.pdf

2. Shane, S. (2015). Lessons learned from the recent US HPAI epornitic. World Poultry [On-line]. Available at: http://www.worldpoultry.net/Health/Articles/2015/9/Lessons-learned-from-the-recent-US-HPAI-epornitic-2693194W/

3. United States Department of Agriculture-Animal and Plant Health Inspection Service (2015). Epidemiologic and other analyses of HPAI-affected poultry flocks: June 15, 2015 Report [Online]. Available at: https://www.aphis.usda.gov/animal_health/animal_dis_spec/poultry/downloads/Epidemiologic-Analysis-June-15-2015.pdf

4. United States Senate (2015). Highly pathogenic avian influenza: the impact on the U.S. poultry sector and protecting U.S. poultry flocks; Hearing before the Committee on Agriculture, Nutrition, and Forestry; United States Senate; One Hundred Fourteenth Congress, First Session. U.S. Government Publishing Office [online]. Available at: http://www.agriculture.senate.gov/imo/media/doc/S.%20Hrg.%20114-153%20-%20HIGHLY%20PATHOGENIC%20AVIAN%20INFLUENZA%20THE%20IMPACT%20ON%20THE%20U.S.%20POULTRY%20SECTOR%20AND%20PROTECTING%20U.S....pdf

BIOSECURITY SELF-ASSESSMENT

K. Takeshita

CDFA-AH Branch

During 2015 HPAI/LPAI Outbreaks, the need for improved biosecurity became apparent. Numerous Federal and State programs try to address biosecurity including NPIP, CEQAP, FDA egg rule and SEFS. To enable the producer/owner to assess/improve their own biosecurity, a Biosecurity Self-Assessment Guide was developed in CA. The Biosecurity Self Assess is divided sections (Figure 1).

Each section is in tabular form divided into multiple rows representing Risk Categories and three columns representing low, moderate and high risk. In each column for each category, an explanation of what a low, moderate and high risk is described for that row (Figure 2.). Simply go down the list and determine the criteria that best fits your current biosecurity practices. Each response is rated as Minimal Biosecurity Risk, Medium Biosecurity Risk, or High Biosecurity Risk.

Minimal Biosecurity Risk. Based on current knowledge, these biosecurity practices are outstanding and you have reduced the risk of introducing infectious disease into your flock. Efforts should be directed toward improving the biosecurity practices that score in the previous categories to meet this level.

Medium Biosecurity Risk. Based on current knowledge, your farm has moderate biosecurity practices in place to prevent introduction of disease. However, there is room for improvement and you may consider consultation with your poultry veterinarian to review these practices and assess the value of making changes to further safeguard your flocks.

High Biosecurity Risk. Based on current knowledge, this biosecurity practice (or lack thereof) puts your flock at an extremely high risk of disease introduction. Consultation with your poultry veterinarian is recommended to determine if your biosecurity protocols in these areas should be or can be changed to better protect your flock and the rest of the industry.

Routine Biosecurity Risk Assessment. This Biosecurity Assessment was distributed at industry meeting and posted on CDFA and industry Web Sites since the middle of 2016. This Guide allows the producer or farm manager the opportunity to assess their current level of on-farm biosecurity. Their answers will provide them with an idea of where there are areas of weakness that require attention or practices that fall below current industry standards.

Biosecurity Risk Assessment During an Outbreak. Using the Biosecurity Self-Assessment, trained assessors will visit sites with birds within three km around an infected premise. At the site they will identify any high risk items and record them on high risk Record Sheet (Figure 3) noting the category (row #) of high risk in first column, description of high risk second column and if photos were taken in third column.

If high risk items are observed, surveillance and epidemiology would be contacted and enhanced surveillance may be initiate until high risk is mitigated.

Dry run on Biosecurity/Permitting Audits poultry sites will be conducted to do actual run through of protocols with CDFA district personnel and with poultry companies to make sure that protocols work, train auditors and give poultry industry to see how the process is going to work. Biosecurity Risk Self-Assessment Guide can be found at https://www.cdfa.ca.gov/ahfss/Animal_Health/BioSpecies/CommercialPoultryBiosecurity.html

REFERENCES

1. Bowes, Victoria, DVM, MSc, ACPV. B.C. 2004. Poultry Industry Enhanced Biosecurity Initiative Producer Self-Assessment Guide. Animal Health Centre, BCMAL. Abbotsford, British Columbia.

2. CDFA Avian Influenza Biosecurity Task Force (2002-2003 Exotic Newcastle Disease Veterans).

3. USDA APHIS VS. 2015. Epidemiologic and Other Analyses of HPAI-Affected Poultry Flocks: July 15, 2015 Report.

4. USDA APHIS VS. July 2015. "Prevent Avian Influenza at Your Farm: Improve Your Biosecurity with Simple Wildlife Management Practices."

5. CDFA. 2015. Industry Biosecurity Plan Example.

6. Select Agent Program Checklist for HPAI.

7. USDA APHIS VS. November 2013 (Draft). "Highly Pathogenic Avian Influenza Standard Operating Procedure: 9. Biosecurity". SOP Number 0009.

8. USDA APHIS VS. Highly Pathogenic Avian Influenza Secure Egg Supply Plan – *Summary of Products and Permitting Requirements*. FAD PReP Foreign Animal Disease Preparedness & Response Plan. Secure Food Supply Plan.

9. BC Poultry Association Biosecurity Committee and Dr. Bill Cox (editor). 2006. BC Poultry Biosecurity Reference Guide.

10. National Flyway Council. June 2015. Surveillance Plan for Highly Pathogenic Avian Influenza in Waterfowl in the United States.

11. Hill, D. Biosecurity in hatcheries. In: Biosecurity in the Poultry Industry. S. Shane, ed. American Association of Avian Pathologists, Keneth square, PA. pp 75-78. 1995.

12. Rosales, G.A., and E. Jensen Special considerations for primary breeders. In: A Practical Guide for Managing Risk in Poultry Production. R.L. Owen, ed. American Association of Avian Pathologists Jacksonville, FL. pp 225-248. 2011.

13. Hofacre, C.L., and G.A. Rosales Prevention and control of disease in primary and multiplier broiler breeder operations. In: Biosecurity in the poultry industry. S. Shane, ed. American Association of Avian Pathologists, Keneth Square, PA. pp 79-84. 1995.

14. Ghazikhanian, G.Y. Prevention and control of diseases in primary and multiplier turkey breeder operations. In: Biosecurity in the poultry industry. S. Shane, ed. American Association of Avian Pathologists, Keneth Square, PA. pp 95-100. 1995.

15. Wojcinski, H. Special considerations for turkeys. In: A Practical Guide for Managing Risk in Poultry Production R.L. Owen, ed. American Association of Avian Pathologists, Jacksonville, FL. pp 249-256. 2011.

16. P., W.D. Principles of disease prevention in commercial turkeys. In: Biosecurity in poultry premises S. Shane, ed. American Association of Avian Pathologists, Keneth Square PA. pp 101-103. 1995.

17. Dekich, M.A. Principles of disease prevention in integrated broiler operations In: Biosecurity in the poultry industry. S. Shane, ed. American Association of Avian Pathologists, Keneth Square, PA. pp 85-94. 1995.

Figure 1

General

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Figure 2. Poultry Facility Biosecurity Risk Assessment Guide.

	Location	Minimal Biosecurity Risk	Medium Biosecurity Risk	High Biosecurity Risk	
2	**Proximity to nearest unrelated commercial poultry operation**	Greater than 2 miles	1/2 mile to 2 mile	Less than 1/2 mile	(1,2)
4	**Proximity to nearest backyard poultry**	Greater than 2 miles	1/2 mile to 2 mile	Less than 1/2 mile	(2)
	Premises Entry/Security				
10	**Perimeter Fencing And Gates**	Complete Perimeter Fence present. Driveway is gated and always locked or guarded	Perimeter fencing and gate present, but not always locked or guarded. Or fence not complete	There is no perimeter fencing or gate	(1)
13	**Vehicle Entry and Disinfection**	Freshly stocked vehicle disinfection station with high pressure sprayer at the gate for all vehicles	Inadequate vehicle disinfection station	No vehicle disinfection station or not used	(2)
	People Entry/Personnel				
34	**Sharing of personnel**	Personnel are dedicated to work on this farm only and not shared with any other farm or off-site facility	Personnel are shared between this farm and other farms of this same company, but not with any off-site facility	Personnel are shared between this farm and a farm of another company, or shared with an off-site facility	(2,3)
35	**Personal Protection Policy**	Shower in policy with disposable or dedicated clothing and footwear before entry	Disposable or dedicated clothing and footwear and required washing/disinfecting of hands before entry (no shower-in policy)	Personnel lack disposable or dedicated clothing and footwear and/or do not practice washing/disinfecting of hands before entry	(1)
42	**Fairs (bird exhibit areas)**	No contact with other birds within 72 hours prior to entry	No contact with other birds within 48 hours prior to entry	No restrictions on contact with other birds	(1,2)
66	**Standing Water, Ponds, or other water bodies on Farm**	Water not allowed to pool or stand for more than 48 hours	Water not allowed to pool or stand for more than 72 hours	Allow standing water and/or ponds on the farm	(4)

Figure 3. "High Risk" Record Sheet.

Row	Description of "High Risk" Deficiency	Photo
66	**There is a drainage ditch with standing about 10' from house 16. Ducks were observed in other areas of standing water**	Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No
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#		Yes/No
#		Yes/No
#		Yes/No
#		Yes/No

ILT IN COMMERCIAL BROILERS HOUSED IN "LAYER LAND": SCENARIO, STRATEGIES, AND SUGGESTIONS

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SUMMARY

Indiana ranks third or fourth in table egg production and number one in duck production. Broiler production in the state is small, but there are commercial broiler farms interspersed within the layer/duck dense counties. The layer operations range from cage free predominantly Amish owned farms to large scale environmental enriched multilevel houses. Infectious laryngotracheitis virus (ILT) is a sporadic issue in the layer flocks, particularly in the fall and early winter. Most layers are vaccinated, generally with the recombinant vaccine, but broilers are not vaccinated. We will be presenting a case study on a broiler farm, a client of the Purdue University Poultry Diagnostic Service, with an outbreak of ILT.

CASE HISTORY

In October, 2016, the managers of the farm contacted our service for investigation of increasing mortality in broiler chickens. The broilers were within a week of processing and were exhibiting signs of respiratory disease. Necropsies performed on-site by farm personnel showed tracheal reddening, hemorrhage, diphtheritic plugs and conjunctivitis. No treatment had been instituted, as the farm was antibiotic free. Nearby layer operations (within five miles) had no reports of mortality or disease. Samples were submitted to the Purdue Animal Disease Diagnostic Laboratory for ILT and HPAI testing.

CASE REPORT

PCR testing on tracheal samples were negative for HPAI and positive for ILT. Virus was isolated and sent to PDRC in Georgia for typing. In the interim, the farm was depopulated, cleaned and disinfected. Biosecurity protocols were reviewed and increased. Results from PDRC showed the isolated virus to be consistent with CEO ILT vaccine.

DISCUSSION

It was determined that a layer farm within two miles of the broiler farm had vaccinated with CEO ILT the week prior to the outbreak. In Indiana, no permits are required for ILT CEO vaccination. The location of the broiler farm was down-wind in a frequently windy area. This type of outbreak is becoming more commonplace in layer dense areas that are endemic for ILT. Options for prevention/control of ILT will be discussed.

RISK OF A POULTRY FLOCK BECOMING INFECTED WITH HPAI-VIRUS DUE TO GARBAGE MANAGEMENT

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ABSTRACT

Garbage management represents a potential pathway of HPAI-virus infection of poultry as multiple poultry premises may share a common trash collection service provider, trash collection site (i.e., shared dumpster for multiple premises) or disposal site (e.g., landfill). The types of potentially infectious or contaminated material disposed of in the garbage vary by poultry industry sector. A survey of representatives from the broiler, turkey, and layer sectors revealed that many potentially contaminated or infectious materials are routinely disposed of in the trash on commercial poultry premises. Garbage management is thus a key component that must be considered in order to evaluate the risk of commercial poultry becoming infected with HPAI-virus.

INTRODUCTION

Garbage trucks coming near the barns was a significant risk factor in a case-control study of egg layer flocks in the 2015 United States (U.S.) HPAI outbreak.(8) In the 2014-2015 outbreak of HPAI H5N2 in the U.S., a case-control study of infected egg layer flocks in Nebraska and Iowa identified garbage trucks coming near the barns as a risk for infection at the farm level (OR=14.7; p <.001).(8) Of note, the frequency with which garbage trucks visited the farms in this study is not known.

Garbage management represents a potential pathway for HPAI-virus infection of a commercial poultry flock as multiple poultry premises may share a common trash collection service provider, trash collection site (i.e., shared dumpster for multiple premises) or disposal site (e.g., landfill). HPAI-virus may enter a poultry premises via contaminated garbage management vehicles or drivers. Notably, prior to 2015, epidemiological trace-back questionnaires in AI outbreaks on commercial poultry farms in the U.S. did not specifically investigate garbage management services as a risk factor.

To more fully evaluate the risk of HPAI infection to commercial poultry via garbage management, we initiated a survey of the poultry industry in order to refine the risk and establish mitigation measures.

METHODS

A convenience sample of veterinarians and other managers in the poultry industry were surveyed between June-August 2016 on standard practices for garbage management on farms that they manage or supervise. Surveys were administered using the online polling service Qualtrics (Qualtrics© 2015 Provo, UT, USA. http://www.qualtrics.com). Some survey questions and answer choices were modified to better match the industry to which it was distributed. Additionally, participants were given the option to decline to answer any question within the survey. The survey was determined to be exempt from University of Minnesota Institutional Review Board review.

RESULTS

A total of 63 surveys were completed. Respondents represented the turkey (n=15), broiler (n=8), and layer (n=40) industry sectors. The types of potentially infectious or contaminated material disposed of in the garbage varied by sector of the poultry industry, and many potentially contaminated or infectious materials were reported to be routinely disposed of in the trash as listed in Table 1. Over half of broiler and turkey sector respondents reported the garbage truck may collect waste from multiple poultry premises before depositing the load at a landfill, while a sizable percentage of respondents in all three sectors reported they did not know if the garbage truck route included other poultry premises. The dumpster or garbage collection area may be located at various locations on a premises (reported proximity to the nearest barn of <100 ft to >250 ft), however only a minority of respondents reported sharing a trash collection location between multiple premises.

DISCUSSION

Respondents identified potential HPAI-contaminated or infectious material (i.e., dead wildlife, poultry carcasses, egg shells, and materials that have contacted poultry) reported to be disposed of in the garbage on poultry premises. Estimates of HPAI-virus concentrations in chicken and turkey secretions, feces, feathers, and other tissues generally range between 103 to107 EID50 per gram or per

milliliter, although higher concentrations have been observed in some cases.(2-6, 13, 14, 18) Additionally, influenza virus survival varies depending on strain and environmental conditions, such as humidity and temperature. Virus persistence is generally longer at cooler temperatures and in more humid conditions. Virus survival on materials that may be disposed of in the garbage has been reviewed elsewhere, such as poultry carcasses, feathers, egg shells, egg trays, wood, steel, glass, and PPE.(10, 11, 17, 19, 20) Thus the potential for HPAI-virus to be present in the garbage and survive in that environment is sufficient to infect a bird should the bird become exposed to that material.

Garbage management contractors used by some turkey and broiler premises have been reported to visit multiple poultry premises on one route before depositing a load at the landfill; thus, HPAI-virus-contaminated garbage from an infected but undetected premises may be present on the truck when it arrives at the next poultry farm. The types of potentially contaminated trash from non-commercial poultry operations (e.g., backyard poultry, processing facilities, live bird markets, etc.) are not known, but are assumed to include materials similar to those reported in garbage from commercial poultry operations. In the Netherlands, poor waste management practices pertaining to liquid waste (e.g., waste water) and solid waste have been identified as potentially increasing the risk of avian influenza (AI) transmission in the neighborhood of infected farms (15). A shared dumpster or common trash collection point for multiple poultry premises, while not commonly used in the poultry industry, represents an additional site of potential cross-contamination between commercial poultry operations related to garbage management.

Garbage trucks and drivers typically do not contact live poultry while completing contracted duties on a poultry premises. Biosecurity recommendations and site-specific biosecurity plans may not stipulate specific biosecurity measures for garbage management drivers; however, it is recommended in recent updates to the National Poultry Improvement Plan guidance that visitors follow procedures to cross the perimeter buffer area (PBA) and line of separation (LOS)(16). If garbage management activities and visits occur outside of the PBA, there is a decreased likelihood of cross-contamination between contaminated garbage management and farm personnel, equipment, or other potential fomites that may access the poultry house.

Representatives of all three industry sectors suggest it is common practice for the dumpster or trash collection point to be located at the entrance or perimeter of the farm. This exact distance to the nearest poultry barn may vary; however, this appears to represent a distance of at least 100 feet to the nearest barn for a majority of respondents. Similar to other third-party contractors, cleaning and disinfection of garbage transport vehicles, pickup routing, and landfill practices may be difficult to control and are not easily influenced by the poultry grower or integrator if utilizing a contractor to haul garbage. An overwhelming majority of respondents in our survey indicated that they do utilize a contractor for some or all of their garbage transport needs.

This survey was limited by a convenience sampling method of participants with significant experience in the poultry industry and subsequent small sample size. Still, the data is informative for the purpose of risk assessment, and serves to illustrate the variations in industry practice and potential differences between poultry sectors that may operate in the same geographic area. As such, the absence of an affirmative response to a high risk activity does not definitively indicate it is not occurring, and that further evaluation of the prevalence of such practices on an industry-wide scale may be warranted based on this exploratory survey.

CONCLUSION

This preliminary survey identified items in garbage that may contain infectious virus, some which may carry high titers of virus. Additionally, the garbage may originate from both commercial and non-commercial (i.e., live poultry markets and backyard flocks) poultry operations. In previous outbreaks of HPAI in non-commercial poultry operations, disposal of mortality in garbage was identified as a risk factor for AI. In an evaluation of risk factors for live bird markets in New York, New Jersey, Pennsylvania, and New England, markets that disposed of dead birds and offal in the trash were 2.4 times more likely to have a repeated presence of LPAI H5 and H7 viruses (OR: 2.4; 95% CI, 1.8-3.4).(9) In an analysis of risk factors associated with H5N1 in backyard poultry in Egypt from 2010-2012, disposing of mortality and poultry feces in garbage piles outside was highly correlated in the regression model ($F=15.7$; $p < 0.0001$)(12). Therefore, if the garbage management services both commercial and non-commercial poultry operations, the risk that the garbage contains HPAI-virus-contaminated material is likely to be higher. The final destination of the garbage and garbage vehicles, such as to a landfill, also can contribute to the risk of HPAI-virus contamination. Landfills may serve as a potential site for cross-contamination as contracted garbage management services for poultry premises may transport garbage to the same landfill; it has been noted that upon arrival at landfills, garbage

management vehicles may drive over previously deposited garbage (D. Halvorson, personal communication, June 2016). This risk likely increases if landfills are used as an off-site disposal method for infected depopulated flocks, which has been reported in previous LPAI outbreaks. (1, 7)

Given that there is potential for HPAI-virus to be associated with garbage management, and the ease with which virus could to be tracked into the poultry house, the risk of a commercial poultry flock becoming infected with HPAI-virus due to garbage management during an outbreak should be considered. Further research is needed to determine prevalence of these practices in different production systems and across geographic regions in the U.S. and to develop appropriate mitigation measures.

REFERENCES

1. Akey, B. L. Low-pathogenicity H7N2 avian influenza outbreak in Virginia during 2002. Avian Diseases 47:1099-1103. 2003.

2. Beato, M. S., M. Mancin, E. Bertoli, A. Buratin, C. Terregino, and I. Capua. Infectivity of H7 LP and HP influenza viruses at different temperatures and pH and persistence of H7 HP virus in poultry meat at refrigeration temperature. Virology 433:522-527. 2012.

3. Bertran, K., D. E. Swayne, M. J. Pantin-Jackwood, D. R. Kapczynski, E. Spackman, and D. L. Suarez. Lack of chicken adaptation of newly emergent Eurasian H5N8 and reassortant H5N2 high pathogenicity avian influenza viruses in the U.S. is consistent with restricted poultry outbreaks in the Pacific flyway during 2014-2015. Virology 494:190-197. 2016.

4. Brahmakshatriya, V., B. Lupiani, J. Brinlee, M. Cepeda, S. Pillai, and S. Reddy. Preliminary study for evaluation of avian influenza virus inactivation in contaminated poultry products using electron beam irradiation. Avian Pathology 38:245-250. 2009.

5. Chmielewski, R., and D. E. Swayne. Avian Influenza: Public Health and Food Safety Concerns. Annual Review of Food Science and Technology 2:21. 2011.

6. Das, A., E. Spackman, C. Thomas, D. E. Swayne, and D. L. Suarez. Detection of H5N1 high-pathogenicity avian influenza virus in meat and tracheal samples from experimentally infected chickens. Avian Dis 52:40-48. 2008.

7. Dunn, P. A., E. A. Wallner-Pendleton, H. Lu, D. P. Shaw, D. Kradel, D. J. Henzler, P. Miller, D. W. Key, M. Ruano, and S. Davison. Summary of the 2001-02 Pennsylvania H7N2 Low Pathogenicity Avian Influenza Outbreak in Meat Type Chickens. Avian Diseases 47:812-816. 2003.

8. Garber, L., K. E. Bjork, K. A. Patyk, T. Rawdon, M. Antognoli, A. Delgado, S. Ahola, and B. McCluskey. Factors associated with highly pathogenic avian influenza H5N2 infection on table egg layer farms in the Midwest, United States, 2015. Avian Diseases. 2016.

9. Garber, L., L. Voelker, G. Hill, and J. Rodriguez. Description of live poultry markets in the United States and factors associated with repeated presence of H5/H7 low-pathogenicity avian influenza virus. Avian diseases 51:417-420. 2007.

10. Guan, J., M. Chan, C. Grenier, D. Wilkie, B. Brooks, and J. Spencer. Survival of avian influenza and Newcastle disease viruses in compost and at ambient temperatures based on virus isolation and real-time reverse transcriptase PCR. Avian Diseases 53:26-33. 2009.

11. Sakaguchi, H., K. Wada, J. Kajioka, M. Watanabe, R. Nakano, T. Hirose, H. Ohta, and Y. Aizawa. Maintenance of influenza virus infectivity on the surfaces of personal protective equipment and clothing used in healthcare settings. Environmental Health and Preventive Medicine 15:344-349. 2010.

12. Sheta, B. M., T. L. Fuller, B. Larison, K. Y. Njabo, A. S. Ahmed, R. Harrigan, A. Chasar, S. A. Aziz, A.-A. A. Khidr, and M. M. Elbokl. Putative human and avian risk factors for avian influenza virus infections in backyard poultry in Egypt. Veterinary microbiology 168:208-213. 2014.

13. Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster. Characterization of Avian H5N1 Influenza Viruses from Poultry in Hong Kong. Virology 252:331-342. 1998.

14. Spackman, E., J. Gelb, L. A. Preskenis, B. S. Ladman, C. R. Pope, M. J. Pantin-Jackwood, and E. T. Mckinley. The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys. Virology journal 7:1. 2010.

15. Ssematimba, A., T. Hagenaars, J. de Wit, F. Ruiterkamp, T. Fabri, J. Stegeman, and M. de Jong. Avian influenza transmission risks: Analysis of biosecurity measures and contact structure in Dutch poultry farming. Preventive Veterinary Medicine 109:106-115. 2013.

16. The National Poultry Improvement Plan. Report of Voting Results on 9- CFR Proposed Changes. In: NPIP 43rd NPIP Biennial Conference. Bellevue, WA. pp 89-92. 2016.

17. Tiwari, A., D. P. Patnayak, Y. Chander, M. Parsad, and S. M. Goyal. Survival of two avian respiratory viruses on porous and nonporous surfaces. Avian Dis 50:284-287. 2006.

18. Toffan, A., M. Serena Beato, R. De Nardi, E. Bertoli, A. Salviato, G. Cattoli, C. Terregino, and I.

Capua. Conventional inactivated bivalent H5/H7 vaccine prevents viral localization in muscles of turkeys infected experimentally with low pathogenic avian influenza and highly pathogenic avian influenza H7N1 isolates. Avian Pathology 37:407-412. 2008.

19. Wood, J. P., Y. W. Choi, D. J. Chappie, J. V. Rogers, and J. Z. Kaye. Environmental persistence of a highly pathogenic avian influenza (H5N1) virus. Environmental Science & Technology 44:7515-7520. 2010.

20. Yamamoto, Y., K. Nakamura, M. Yamada, and M. Mase. Persistence of avian influenza virus (H5N1) in feathers detached from bodies of infected domestic ducks. Applied and Environmental Microbiology 76:5496-5499. 2010.

Table 1 Survey results of material disposed of in the garbage on premises in the broiler, turkey, and layer industries.[1]

Item	Broiler Sector (n=8 respondents)	Turkey Sector (n=15 respondents)	Layer Sector (n=39 respondents)
Dead wildlife/wild birds	Yes (1/8)	Yes (5/15)	Yes (1/39)
Rodents	Yes (3/8)	Yes (5/15)	Yes (10/39)
Mortality or poultry carcasses	No (0/8)	Yes (1/15)	Yes (9/39)
Eggs or egg products[2]	Yes (1/8)	Yes (1/15)	Yes (8/39)
Manure	No (0/8)	No (0/15)	Yes (1/39)
Spilled feed	Yes (2/8)	Yes (8/15)	Yes (7/39)
Disposable chick transport boxes[2]	Yes (4/8)	Yes (4/15)	Yes (24/39)
Used needles/syringes/diagnostic supplies that have contacted birds[2]	Yes (1/8)	Yes (5/15)	Yes (14/39)
PPE (boot covers, gloves, coveralls, etc.)	Yes (8/8)	Yes (14/15)	Yes (36/39)
Feathers	No (0/8)	Yes (2/15)	Yes (4/39)
Offal	No (0/8)	No (0/15)	No (0/39)
Equipment or supplies from inside barns[3]	Yes	Yes	Yes (22/39)
Household garbage from farm manager or any other residence [3]	--	Yes	Yes (20/39)
Trash associated with waterfowl hunting[3]	--	--	No (0/39)
Garbage from processing operation[3]	--	--	Yes (23/39)
Lunch room and restroom garbage[3]	--	--	Yes (37/39)

[1]Yes indicates materials disposed of in the garbage by one or more survey respondents within each industry. In parenthesis, numerator indicates number of survey respondents reporting disposal of item and denominator indicates total number of respondents.

[2]Language of selection choice modified in survey distributed to representatives of layer industry.

[3]Item only explicitly asked in survey distributed to representatives of layer industry. Yes in the broiler and turkey industries for these items represent at least one write-in response indicating disposal of that item.

CONTRIBUTION OF THE ENVELOPE-ASSOCIATED AND THE POLYMERASE-ASSOCIATED PROTEIN GENES IN NEWCASTLE DISEASE VIRUS VIRULENCE

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SUMMARY

The virulence of Newcastle disease virus (NDV) is a wide variation and determined by multiple genetic factors. In the present study, we evaluated the roles of the envelope-associated and polymerase-associated protein genes to NDV virulence individually or in combination by exchanging genes between the velogenic genotype VII strain SG10 and the lentogenic genotype II strain LaSota. Sixteen chimeric viruses were recovered and their pathogenicities and replication kinetics were evaluated. The results confirmed that the envelope-associated proteins (F and HN) contributed greatly to NDV virulence. However, for the polymerase-associated proteins, the NP, P or L gene individually had little effect on virulence, while transfer of these three genes in combination significantly affected the virulence of the chimeric virus, especially when the L gene was involved. Our results indicated that the polymerase L protein was another major contributor to NDV virulence when combined with the homologous NP and P proteins. We also investigated the viral RNA synthesis in NDV minigenome systems to assess the interaction between the NP, P, and L proteins in vivo, which showed that the activity of the polymerase-associated proteins were directly related to viral RNA transcription and replication. Our findings provide the basis for furthering understanding of the virulence factors associated with NDV and other paramyxoviruses.

INTRODUCTION

NDV is a member of the genus *Avulavirus* within the family *Paramyxoviridae* and its genome is a non-segmented single-stranded RNA molecule of about 15 kb in length (2). The genome encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large polymerase protein (L).

Based on their pathogenicity in chickens, NDV strains can be categorized into three different pathotypes: lentogenic, mesogenic and velogenic (4). The virulence of NDV is a complex trait and is determined by multiple factors. The cleavage site of the F protein was known as the major virulence determinant, and some other protein genes have also been shown to contribute significantly to NDV virulence (1). However, there are examples of incongruity between different protein genes and virulence, and the contribution of these genes to virulence may be related to the particular NDV strain involved (1, 4).

Genotype VII NDV is the predominant virus currently circulating in the world and it has been revealed to have some new features and a higher pathogenicity in chickens compared with other virulent strains (5). However, how the contribution of the viral proteins of genotype VII strains to the virulence and pathogenicity of NDV remains poorly understood.

In this study, we evaluated the contributions of the envelope-associated and polymerase-associated protein genes in NDV virulence individually or in combination between the velogenic genotype VII strain SG10 and the lentogenic genotype II strain LaSota using several different methods, including MDT, ICPI, replication kinetics in vitro and in vivo, pathogenicity in four-week-old chickens and the RNA synthesis in NDV minigenome.

MATERIALS AND METHODS

Construction and recovery of recombinant chimeric viruses. Virus rescue was performed as described previously (3).

Virulence of the chimeric viruses. The virulence of recombinant viruses was determined by the MDT and ICPI test.

Viral growth kinetics. The growth kinetics of the viruses were determined under multiple-cycle growth conditions in a chicken embryo fibroblast cell line (DF-1) cells as described previously (3).

Replication and pathogenicity of the viruses in 4-week-old chickens. Four viruses, rSGLaNPPL and rLaSGNPPL, and their parent strains (rSG10 and rLaSota) were used to determine the pathogenicity in chickens by inoculating each bird with 106EID50 of virus through the natural route of infection.

Viral RNA synthesis in NDV minigenome systems. Two NDV minigenome systems (pLaSota-FLuc and pSG10-FLuc) were constructed and the dual luciferase assay was carried out.

RESULTS

Recovery of recombinant viruses. 16 chimeric viruses in which the F, HN and NP, P, L ORF genes were replaced individually or in combination between strain rSG10 and rLaSota were recoveried.

Virulence of the recombinant viruses. The MDT and ICPI values for all rescued viruses showed the envelope-associated protein genes had a marked effect on the viral virulence, and the L protein associated with the homologous NP and P proteins contribute to NDV virulence. The MDT/ICPI values were showed as follows: rLaSota, >120 h/0; rLaSGNPPL, >120 h/0.63; rLaSGFHN, 79 h/1.49; rSG10, 45 h/1.86; rSGLaNPPL, 89 h/1.32; rSGLaFHN, >120 h/0.86.

Growth kinetics of the mutant viruses. The growth kinetics of the viruses were evaluated using multicycle growth curves in DF-1 cells (Fig. 1). The results showed that among the polymerase-associated protein, the NP and L proteins had an obvious influence on the level of virus replication.

Replication and pathogenicity of chimeric viruses in four-week-old chickens. The pathogenicity of four viruses were evaluated in four-week-old SPF chickens by inoculating each bird with 106 EID50 of virus by the oculonasal route. The results showed that the rSG10 developed severe illness and a 100% mortality by day five, while others had no death. Two chickens from each group were sacrificed daily for virus titration, which released rLaSGNPPL with an increased replication ability compared with rLaSota and rSGLaNPPL indicating a decreased replication ability of rSG10 (Fig. 2A).

Viral RNA synthesis in NDV minigenome systems. The dual luciferase assay was carried out to further detect whether viral RNA transcription and replication correlated with the intrinsic activity of the polymerase-associated proteins (Fig. 2B), which revealed that the activity of the SG10 replication complex was higher than that of the LaSota replication complex, and the combined action of all three homologous replication proteins was required to reach the highest level of activity.

DISCUSSION

In the present study, the velogenic genotype VII strain SG10 and the lentogenic genotype II strain LaSota were chosen to evaluate the roles of the envelope-associated and polymerase-associated protein genes in NDV virulence individually or in combination. We first confirmed the F and HN envelope glycoproteins played an important role in NDV virulence. We next evaluated the contributions of the polymerase-associated protein genes (NP, P and L), there being several examples of incongruity between different protein genes and virulence, as well as evidence that the contribution of these genes to virulence might be related to the particular NDV strain involved (1, 2, 4). Our result indicated that the polymerase L protein of NDV was the next most prominent individual contributor and was sometimes augmented by the homologous NP and P proteins.

We further determined the pathogenicity of two chimeric viruses (rSGLaNPPL and rLaSGNPPL) and their parental viruses in four-week-old SPF chickens inoculated via a natural infection route. There was a marked difference in the pathogenicity and tissue tropism between the parental virus and its chimeric viruses. The rSG10 caused an obvious 100% mortality and a high level of tissue titers, whereas the chimeric virus rSGLaNPPL maintained a low pathogenicity. In contrast, the rLaSGNPPL caused an increased replication efficiency in more of the tissues compared with rLaSota. We also using the NDV minigenome systems proved that the ribonucleoprotein complex (RNP), as the minimum structural unit, is required for viral transcription and replication, and the function is carried out by the polymerase complex in its entirety, not by individual proteins. In summary, our study further confirmed that the virulence of NDV is determined by multiple viral proteins. The F and HN proteins are the major contributor to virulence, and the L protein as well as NP and P protein also have an obvious influence on virulence.

ACKNOWLEDGMENTS

This study was supported by the Beijing Agriculture Innovation Consortium of Poultry Research System (BAIC04-2016).

REFERENCES

1. Dortmans, J. C., Koch, G., Rottier, P. J., and Peeters, B. P. Virulence of Newcastle disease virus: what is known so far? Vet. Res. 42, 122. 2011.

2. Kim, S. H., Subbiah, M., Samuel, A. S., Collins, P. L., and Samal, S.K. Roles of the fusion and hemagglutinin-neuraminidase proteins in replication, tropism, and pathogenicity of avian paramyxoviruses. J. Virol. 85, 8582–8596. 2011.

3. Liu, M.M., J.L. Cheng, X.H. Yu, Z.M. Qin, F.L. Tian, and G.Z. Zhang. Generation by reverse genetics of an effective attenuated Newcastle disease

virus vaccine based on a prevalent highly virulent Chinese strain. Biotechnol Lett. 37:1287-1296. 2015.

4. Paldurai, A., Kim, S. H., Nayak, B., Xiao, S., Shive, H., Collins, P. L., et al. Evaluation of the contributions of individual viral genes to Newcastle disease virus virulence and pathogenesis. J. Virol. 88, 8579–8596. 2014.

5. Zhang, Y. Y., Shao, M. Y., Yu, X. H., Zhao, J., and Zhang, G. Z.. Molecular characterization of chicken-derived genotype VIId Newcastle disease virus isolates in China during 2005-2012 reveals a new length in hemagglutinin-neuraminidase. Infect. Genet. Evol. 21, 359–366. 2014.

(The full-length article will be published in Frontiers in Microbiology)

Figure 1. Growth kinetics of the viruses reporter gene expression (B)

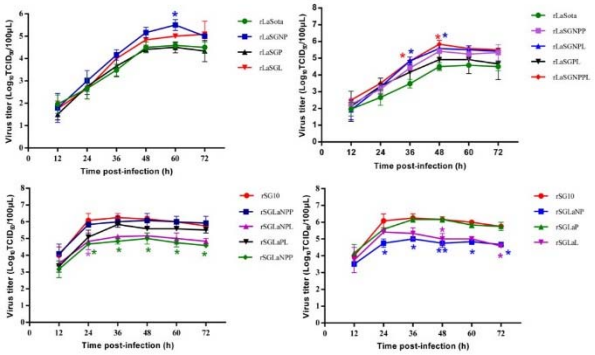

Figure 2. Replication of the viruses (A) and relative.