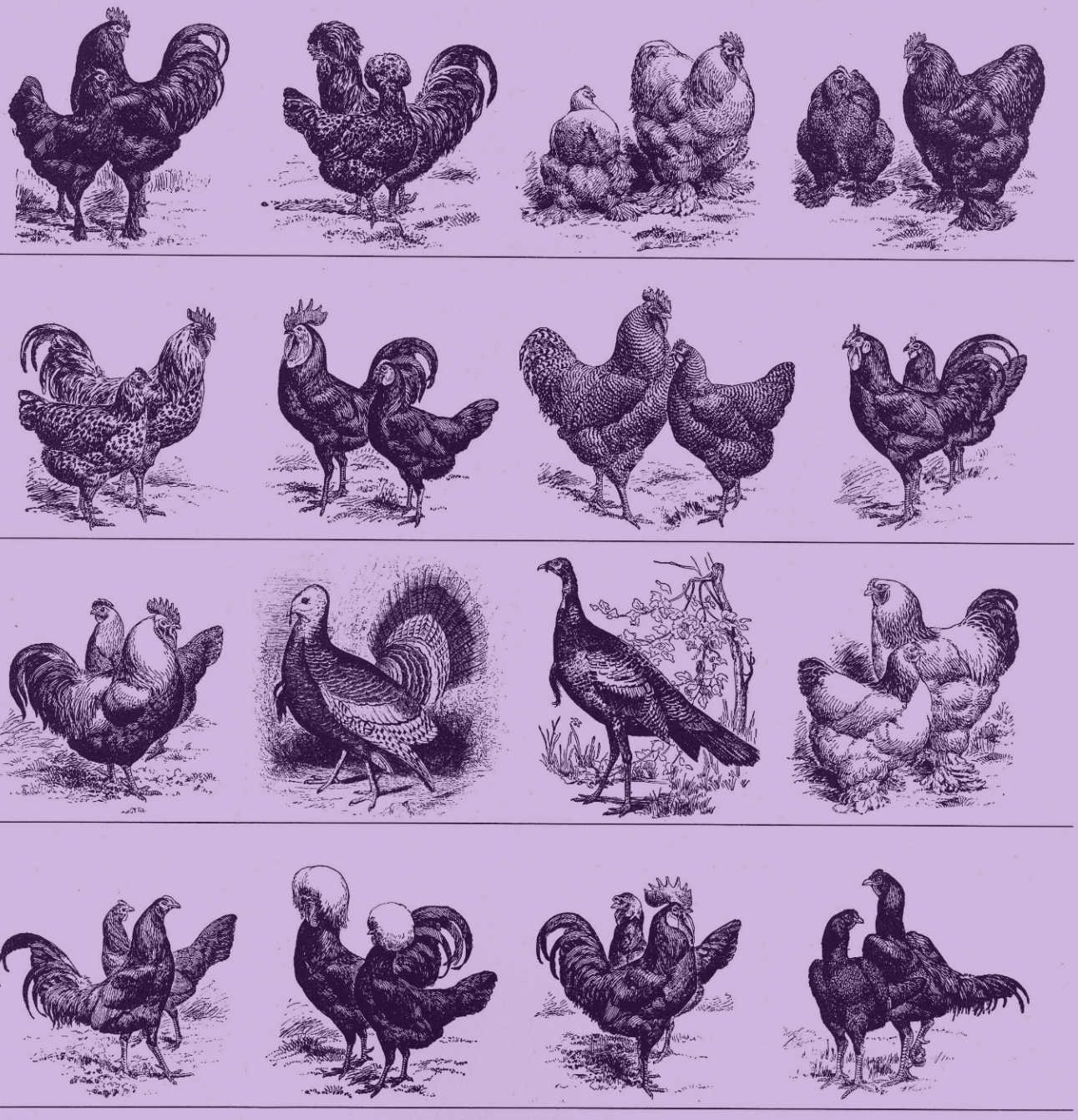
PROCEEDINGS OF THE SEVENTY-SECOND WESTERN POULTRY DISEASE CONFERENCE

March 13-15, 2023 Sacramento, California

WESTERN POULTRY
DISEASE CONFERENCE



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THE 72nd WESTERN POULTRY DISEASE CONFERENCE DEDICATION

PETER ROBERT 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 recounted, "My first job as a virologist was working with duck virus hepatitis at the Animal Health Trust near Newmarket in East Anglia. I was there until 1984. It was the year I hit 40 in September and by the end of that month I was out of work as my research funding had run out. The omens of 1984!"

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.

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 reminisced, "I was very sad when the Fresno lab closed in 2009 as there are so many good times and successes to be remembered there, including the NDV outbreak, identifying Hepatitis E virus, isolation of very virulent IBDV, isolation of WNV and many isolations of AIV."

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. That same year he was honored by the Pacific Egg and Poultry Association as the recipient of the 2013 Scientist of the Year award.

Peter enjoyed swimming, gardening, studying photography, yoga, and traveling. He also became involved with local government by serving as a trustee on the Fresno Mosquito and Vector Control District Board.

Peter's passing on November 7, 2022 has indeed left a void in the profession and in the lives of those who were acquainted with him. The 72nd Western Poultry Disease Conference is honored to dedicate this year's meeting to Dr. Peter Woolcock.

THE 72nd WESTERN POULTRY DISEASE CONFERENCE SPECIAL RECOGNITION AWARD

CHARLES CORSIGLIA

Charles obtained a Bachelor of Science in biology and animal sciences from the California Polytechnic State University in 1989 and consequently received his DVM from Auburn University College of Veterinary Medicine in 1993. After veterinary school he practiced veterinary medicine at a mixed animal practice hospital in Bardstown, KY for two years. He received his Master of Avian Medicine from the University of Georgia in 1996 and became a diplomate in the American College of Poultry Veterinarians in 1997.

His first poultry-related job was at Plantation Foods / Cargill in Waco, Texas where he practiced poultry medicine from 1997 to 2001. After that he joined Foster Farms where he is currently Director of Veterinary Services.

During his 30 years' career, 26 of which have been in poultry medicine, Dr. Corsiglia has touched his colleagues and students' lives by his expertise in poultry medicine and in his life experiences. Stories like "you will kill one or two cows when you start your practice" are proof that he is willing to share good and bad experiences as learning opportunities. He has played a huge role in leadership positions. Proof of that is being the chair of the California HPAI Task Force and the California Animal Health and Food Safety Laboratory advisory board. He is constantly involved in education through teaching lectures as part of the poultry medicine block at UC Davis as well as receiving veterinary students for field trips and externships. He is a very approachable person who is capable of orienting one's career and life in the same conversation.

It is a pleasure and privilege to present Dr. Charles Corsiglia the 72nd Western Poultry Disease Conference Special Recognition Award.

SPECIAL ACKNOWLEDGEMENTS

The 72nd Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and help defray some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international meeting. Many companies and organizations have once again given substantial financial support, including some that also send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

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

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

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

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

We wish to express a very special thanks to BK Association Management for managing our conference, for providing the Proceedings on the WPDC Foundation web site, and facilitating RACE-approved CE credit for attendance at the WPDC!

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WPDC FOUNDATION BOARD OF DIRECTORS

72nd WPDC PROCEEDINGS

Please note that the proceedings of the 72nd 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 72nd WPDC are available in electronic format only. They can be downloaded from the WPDC Foundation website (www.wpdcfoundation.org).

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	P. Cortez	Ernesto Soto	Hugo Medina	
39th ANECA	Néstor Ledezma M.	Ernesto Soto	Benjamin Lucio Martínez	
64th WPDC – 2015	Ernesto Soto	Shahbaz Haq	Bruce R. Charlton	David Willoughby
65th WPDC – 2016	S. Haq	Susantha Gomis		
66th WPDC – 2017	S. Gomis	C. Gabriel Sentíes-Cué	Richard McCapes	Peter Woolcock Richard P. Chin
67th WPDC – 2018	C.G. Sentíes-Cué	Rodrigo A. Gallardo		David D. Frame
68th WPDC – 2019	R. Gallardo	Sarah Mize		Gregg J. Cutler
44th ANECA	Ricardo Cuetos Collado	Maritza Tamayo		
69th WPDC – 2020	S. Mize	Simone T. Stoute		Mark C. Bland
70th WPDC – 2021	S. Stoute	Lynn G. Bagley	Walter F. Hughes	H. L. Shivaprasad
71st WPDC – 2022	S. Stoute	Simone T. Stoute		
72nd WPDC– 2023	S. Stoute	Carmen Jerry	Peter Woolcock	Charles Corsiglia

MINUTES OF THE 71st WPDC ANNUAL BUSINESS MEETING

Secretary-Treasurer Gallardo called the meeting to order on Monday, April 14, 2022, at 4:30 PM PST. Executive committee members present are Rodrigo Gallardo, Simone Stoute, Nancy Reimers, Shelly Popowich, and Ana Paula da Silva. David Frame is absent.

APPROVAL OF 70th WPDC BUSINESS MEETING MINUTES

Minutes of the 70th Western Poultry Disease Conference business meeting are unanimously approved.

ANNOUNCEMENTS

The WPDC is now officially a nonprofit organization with 501(c)(3) status and has a functioning board of directors. Their specific duties are David Frame, proceedings editor; Ana Paula da Silva, student program chair; Nancy Reimers, contributions chair; Simone Stoute, president and agenda chair for 2022; Shelly Popowich, executive secretary; and Rodrigo Gallardo, secretary-treasurer.

The WPDC Constitution is reviewed, and the following items are discussed. A local arrangements chair-elect will be appointed on an as-needed basis. The Special Recognition Award is to be modified so that it isn't always given posthumously, but rather that there is a more general membership involvement. Any conference dedications are to be exclusively designated by the WPDC Board of Directors. The A.S. Rosenwald lecture is to be given by someone who has had a significant career commitment in poultry extension.

REPORT OF THE SECRETARY-TREASURER

Secretary-Treasurer Rodrigo Gallardo announces that there are 242 people registered for the 2022 meeting. Of those, 146 are registered in-person. In contrast, there were 144 people registered online for the 2021 conference. Registration income for the 2022 conference was $22,950 with sponsorship donations amounting to approximately $31,000. There were 22 contributors to this year's meeting. Entitlement levels are revised to better clarify the information for potential sponsors. Dr. Gallardo notes that the cost of this year's meeting is approximately $70,000.

REPORT OF THE PROGRAM CHAIR

The program chair report is given by Dr. Simone Stoute. There were 62 titles submitted for oral presentations. Besides those submitted, there were ten withdrawals along with four rejections. Four keynote speakers were invited to present. Seven posters were exhibited. Students will be encouraged to participate and submit papers. In future years, it is anticipated that a QR code will be available for uploading meeting agendas and presentations.

REPORT OF THE STUDENT PROGRAM CHAIR

Dr. Ana Da Silva, who serves as the student program coordinator, announces that eight case reports and six basic research student presentations were given. Seven people were asked to serve as an ad hoc review committee to evaluate the student submittals. A $500 award was given to the best student presentations in these categories.

REPORT OF THE PROCEEDINGS EDITOR

There were 70 manuscripts published. As we continue to explore new methods of collecting future manuscripts, challenges of communication must be resolved along the way. This year, there was some confusion regarding who to contact about manuscript submittals. We anticipate this problem to be cleared up as we move forward.

WPDC EXECUTIVE COMMITTEE

Dr. Carmen Jerry is nominated for program chair of the Sacramento meeting in 2023. For the 2024 conference in Salt Lake City, Shelly Popowich is nominated as the program chair elect. Dr. Nancy Reimers moved that nominations be closed. Motion is seconded by Dr. Gregg Cutler. All nominees are approved unanimously.

FUTURE MEETINGS

2023: The contract with the Holiday Inn Sacramento Downtown – Arena is secured for the 72nd WPDC to be held March 12-15.

2024: The 73rd WPDC will be held in Salt Lake City. It is anticipated that we will have the meeting in mid-April.

2025: Options are being explored for an appropriate venue.

NEW BUSINESS

Because of increasing costs, raising of registration fees for future meetings is discussed. It is proposed that the following fee schedule be adopted:

Non-presenter: $300
Non-presenter (on site): $350
Presenter: $250
Student: $100
Luncheon: dependent on local charges

The use of social media platforms, such as Facebook, Twitter, and LinkedIn, be considered for greater visibility for the WPDC. The possibility of including an ancillary meeting focused on production-oriented topics is discussed.

ADJOURNMENT

It is moved by Dr. Cutler and seconded by Dr. Reimers that the meeting be adjourned. Voting is unanimous in the affirmative. Dr. Gallardo adjourned the 71st WPDC annual business meeting at 5:05 PM PST.

TABLE OF CONTENTS

Underlined name denotes submitting author.

MYCOPLASMA GALLISEPTICUM WORLD RECORD ANTIBIOTIC RESISTANCE IS CLAIMED BY VIETNAMESE ISOLATE

R. Achari[1], Z. Kreizinger[2], M. Gyuranecz[2], and C. Morrow[1]

[1]Bioproperties, Ringwood, Australia
[2]Molliscience, Budapest, Hungary

SUMMARY

As part of a continuing survey of antibiotic sensitivity of *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) in Asia samples were submitted to our Hungarian lab. These samples had been collected using the same methodology as in Morrow and others (3) but only one bird was swabbed per sample. An isolate (J2) was obtained by immediately filtering (0.45 µm disposable filter) the sample in media at the farm. Isolation took about 3 weeks in the laboratory. At the time of sampling the flock had recently been medicated for producing very poor quality progeny.
J2 was isolated from a native chicken breeder in Vietnam that had high Minimum Inhibitor Concentration (MIC) to enrofloxacin (>16 µg/mL), erythromycin (>32 µg/mL), spiramycin (>16 µg/mL), tilmicosin (>64 µg/mL), tylosin (4 µg/mL), lincomycin (32 µg/mL), spectinomycin (>16 µg/mL). It was partially resistant to oxytetracycline (8 µg/mL). It had low MICs to tiamulin (0.3 ug/mL), tylvalosin (0.5 µg/mL) and florfenicol (2 µg/mL).

Often MIC of avian mycoplasma isolates does not correlate with lack of clinical response to treatment and PCR semi-quantitative results (comparison of C_ts at beginning and end of therapy). The high MIC to enrofloxacin is very common now in SE Asia (3). The results of the initial survey were similar to the MICs seen in Europe in recent samples (2).

There are big differences in medicating habits between Europe and SE Asia. Polypharmacy and rapid rotation being more common in SE Asia and this does not seem to be reflected in the results of surveys. Or perhaps fast rotation prevents resistant strains from becoming permanently established. Biases can also be introduced by sampling criteria (1) making "resistant" isolates less likely to be included in surveys.

Perhaps strains that have developed resistance are outcompeted in vitro during isolation especially with pooling. Further studies to characterize this isolate will look at SNPs associated with resistance, plasmid carriage and efflux-based resistance.

This strain with a wide range of acquired resistance might be what is being fought in the field rather than what has been previously characterized in the laboratory. To our knowledge this MG is the most "resistant" avian mycoplasma described.

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3. Morrow, C.J., Kreizinger, Z., Achari, R.R., Bekő, K., Yvon, C., Gyuranecz, M. (2020): Antimicrobial susceptibility of pathogenic mycoplasmas in chickens in Asia. Veterinary Microbiology, 250, art. no. 108840. DOI: 10.1016/j.vetmic.2020.108840.

EFFICACY OF SEROTYPE 1 LOW PASSAGE RISPENS AND CHIMERA MAREK'S DISEASE VACCINES ALONE OR IN COMBINATION WITH RECOMBINANT HVT-IBD VACCINES IN SPF BIRDS

I. Alvarado[1], R. Koopman[2], H. Swam[2], and J. Petit[2]

[1]Merck Animal Health, Watkinsville, USA. [2]MSD, Boxmeer, Netherlands

SUMMARY

The efficacy of two Marek's disease serotype 1 vaccines (a low passage Rispens (LP) and a Chimera strain), when administered alone or in combination with HVT-IBD recombinant vaccines, against RB1B challenge was evaluated. SPF Leghorn chicks were equally divided and randomly assigned to the following treatment groups: 1) Non-Vaccinated/Challenged, 2) LP Rispens 3) LP Rispens & HVT-ND-IBD, 4) Chimera, and 5) Chimera & HVT-IBD. Birds in all the groups were challenged intramuscularly at 5 days of age and were observed during 70 days for the presence of clinical signs and mortality. At the end of the study, all the birds were evaluated for the presence of macroscopic lesions. Relative protection percentages (RPP) were calculated according to the European Pharmacopoeia. RPPs of 0%, 90%, 97%, 63% and 90% were observed in groups 1 to 5, respectively. SPF birds vaccinated with the LP Rispens vaccine alone or in combination with HVT-ND-IBD showed superior protection against challenge.

EXPERIMENTAL DESIGN

Birds. A total of 150 one-day-old SPF chickens were randomly divided in 5 treatment groups and placed in separate negative pressure isolators (n=10/isolator) with feed and water at libitum. At day of age, birds in the respective groups were subcutaneously vaccinated with HVT vectored, MDV Rispens and/or Chimera vaccines as shown in Table 1.

Challenge. At 5 days of age, chickens in all the groups were challenged intramuscularly with the very virulent Marek Disease virus RB1B strain (26 CID50/0,2mL). The challenge dose was adjusted following the guideline of the European Pharmacopoeia Monograph 0589, MD vaccine (live), Section 2-3-1 to cause mortality and/or severe macroscopic lesions in at least 70% of the Non-vaccinated/Challenged chickens within 70 days post-challenge.

Detection and differentiation of MDV Rispens and field (challenge) strains. Spleen and feather pulp samples obtained from 10 birds per group during the trial (euthanized before the end of the study or at the end of the study) were placed on FTA cards for detection and differentiation of Rispens CVI988 and field (challenge) strains. The extraction of DNA from FTA cards was performed with the MagNA Pure 96 robot according to SOP: 256220-106. Kylt®. MDV & Rispens DIVA Detection Kits comprise a Multiplex Real-Time PCR for the detection of viral DNA of Marek Disease Virus and the differentiation between field strains and the vaccine strain Rispens CVI988.

Efficacy against challenge. According to the requirements of the European Pharmacopoeia, protection against challenge is present if at least 70% of the vaccinated birds show no clinical signs or mortality associated with Marek's disease. Relative percentage of protection (RPP) for the vaccinated groups was calculated using the following formula:

$$RPP = \frac{V1 - C2 \times 100}{100 - C}$$

1 V = Percentage of challenged vaccinated chickens that survived until the end of the observation period without notable clinical signs or macroscopic or microscopic lesions of Marek's disease.

2 C = percentage of challenged control chickens that survived till the end of the observation period without notable clinical signs or macroscopic or microscopic lesions of Marek's disease.

RESULTS

Detection and differentiation of MDV Rispens and field (challenge) strains. As expected, all the spleen and feather pulp samples from the Non-vaccinated/Challenged group were positive for the field (challenge) strain.

In the spleen samples retrieved from the LP Rispens, LP Rispens + HVT-ND-IBD and Chimera vaccinated groups a mixture of positive reactions to the field strain were detected, with the presence of the MDV Rispens and double infection. The group vaccinated with Chimera + HVT-IBD vaccines showed an unexpected result with detection of the field strain but no Rispens or double infection.

In the feather pulp samples retrieved from LP Rispens, LP Rispens + HVT-ND-IBD and Chimera + HVT+IBD vaccinated groups, a mixture of positive reactions was also detected, with the presence of the field strain, the MDV Rispens and double infection. In the feather pulp samples retrieved from the Chimera vaccinated group either a field strain or a double infection but no Rispens was detected.

Efficacy against challenge. The level of protection provided by different Marek's vaccines against challenge with the vvMDV RB1B strain is summarized in Table 2. Chickens in the Non-vaccinated/Challenge group (positive control) showed characteristic clinical signs of MDV post challenge. Chickens in this group were euthanized on the tenth- or eleventh-day post challenge. In contrast, chickens in the different vaccinated groups showed moderate to high protection. The highest RPP (96.7%) was observed in the LP Rispens + HVT-ND-IBD vaccinated group, followed by the Chimera + HVT-IBB (90%) and LP Rispens (90%) groups. The lowest level of protection (63%) was observed in the group vaccinated with the Chimera vaccine alone. The level of protection observed in this group did not meet the minimum requirements of the European Pharmacopoeia.

CONCLUSION

The data obtained from this study indicates that all vaccinated groups except for the Chimera group, showed high levels of protection against challenge at day 5 with the vvMDV RB1B strain. The data obtained from this study initially indicates that the Kylt® MDV & Rispens DIVA qPCR kit could be used to detect DNA from MDV Rispens and field strains from spleen and feather pulp impressions on FTA cards. However, further evaluations of technical issues with cross-reactivity with the RB1B challenge strain, potentially leading to false positives on Rispens detection, should be performed.

Table 1. Summary of the experimental design with treatment groups, age and route of challenge and evaluation period.

Group	# Birds	Treatment Groups & Challenge		
		Day 1 vaccination subcutaneous	Day5 challenge vvMDV RB1B	End of trial Day 75 (70dpc)
1	30	Non-Vaccinated	Intramuscular	Daily Observations Euthanasia/Clinical Observation
2	30	LP Rispens		
3	30	LP Rispens & HVT-ND-IBD		
4	30	Chimera		
5	30	Chimera & HVT-IBD		

Table 2. Percentage of protection provided by different Marek's disease vaccines in SPF Leghorn chickens vaccinated subcutaneously at day of age and challenge intramuscularly at five days of age.

Group	Vaccination Day-old SC	Protection	
		# Protected/Total	Protection %
1	Non-Vaccinated/Challenged	0/30	0
2	LP Rispens	26/29	90
3	LP Rispens & HVT-ND-IBD	29/30	97
4	Chimera	19/30	63
5	Chimera & HVT-IBD	27/30	90

OVERVIEW OF THE EFFECTS OF *ENTEROCOCCUS CECORUM* EXPOSURE DURING LATE EMBRYOGENESIS ON SYSTEMIC DISEASE AND EARLY PERFORMANCE IN BROILER CHICKENS

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ABSTRACT- MANUSCRIPT

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EVALUATING A CHALLENGE MODEL TO UNDERSTAND THE PATHOGENESIS OF SPOTTY LIVER DISEASE (SLD) CAUSED BY *CAMPYLOBACTER HEPATICUS* IN CHALLENGED SPF CHICKENS

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ABSTRACT

Spotty liver disease (SLD) caused by *Campylobacter hepaticus* has emerged as an important cause of disease in table egg layers worldwide. Disease associated with *C. hepaticus*, causes focal lesions on the livers of infected birds, reduced egg production, reduced egg size and increased mortality. The transmission route is not well understood, but likely the fecal-oral route. The objective of this study was to develop a challenge model to understand the pathogenesis of *C. hepaticus* in challenged birds. 43 SPF chickens, 17 weeks of age, were divided into two groups; one group (n = 35), in which 51% of the birds were orally challenged with *C. hepaticus* and one negative control group (n=8). A dose of 10^7 cfu/mL of *C. hepaticus* was given on day 1, day 4, and day 7 using an oral gavage needle. Bile and liver samples from challenged and non-challenged naïve exposed birds were collected at day 9, 15, 20 and 27 post challenge to evaluate presence of *C. hepaticus* gross lesions and detection via culture and PCR. Results showed that challenged birds were able to develop mild to moderate multifocal liver lesions resembling SLD. Also, severe lesions were evident in the naïve, exposed non-challenged birds. Positive bacterial culture and colony Polymerase Chain Reaction (PCR) positives were found in challenged birds. Results from direct PCR of liver samples were able to detect a greater number of *C. hepaticus* samples than culture and colony PCR of bile isolates alone. Results showed that *C. hepaticus* could be passed horizontally as over 29% of the naïve non-challenged birds were positive on PCR for *C. hepaticus* post introduction to birds that were challenged. Overall, this study found that this challenge model for *C. hepaticus* was successful, which could help the layer industry to understand the pathogenesis of *C. hepaticus.*

INTRODUCTION

Spotty liver disease (SLD) has emerged as an important cause of disease in table egg layers and layer breeders in the United Kingdom (UK), Australia, Jordan, and in the United States (US). In the US it has been detected in the midwestern and southern states (1–5). The organism implicated in SLD, *Campylobacter hepaticus*, has been reported as the causative organism resulting in multifocal lesions on the livers of infected birds which has resulted in reduced egg production, reduced egg size and increased mortality of highly valuable hens. Recently, *Campylobacter bilis* has also been isolated from chickens with spotty liver disease and has been suggested as a second *Campylobacter* species causing SLD in poultry (6).

C. hepaticus is a Gram-negative organism that grows under microaerphilic conditions at 37-42°C, has an S-shaped cell morphology with a single bipolar flagella. The colony morphology is cream-colored, flat-spreading, and has a wet appearance (7). The disease affects hens around peak production (26 to 30 weeks of age), but it has been reported in birds as young as 25-26 weeks of age (8). Of most significance, is the emergence of *C. hepaticus* in free range birds, although the organism has also been documented in caged layers and broilers (1). Necropsy of diseased birds show characteristic multifocal spots on the liver of 1-2 mm in diameter, fibrinous perihepatitis, and splenomegaly with mottling (9). Strains of *C. hepaticus* implicated in disease in the US appear to be highly similar strains implicated in disease in the UK and Australia (1). In the US, there are only three published complete genome sequences of *C. hepaticus* that can potentially be used for vaccine development (2,3,10). Currently, there is limited information on mortality rates as a result of *C. hepaticus* but rates as high as 1-4% have been reported with total mortality as high as

10% (11,12) in UK and Australia. Decreased egg production is a significant concern with losses in the range of 10-25% being reported (13).

C. hepaticus is likely similar to the disease first described by Peckham in 1958 (14) as avian vibrionic hepatitis and disappeared (13) with the gradual conversion to cage rearing. The re-emergence of SLD suggests that cage-free and free-range may be contributing to its reemergence (13) as there is a greater risk for fecal-oral transmission. SLD may occur year-round, and further outbreaks within the same flock when birds are in peak production are possible (8). The potential impact of *C. hepaticus* in breeders is currently unknown, as there have been no studies to measure the prevalence or obtain baseline data on the organism in production birds in Georgia, one of the US's top producers of poultry. Of greatest concern is the decrease in egg production and egg quality which may continue over the life of the flock. However, affected birds may not show overt signs of disease with subclinical disease being possible, resulting in low overall mortality over the life of the flock (13). Anecdotal reports suggest that although birds are infected with *C. hepaticus*, not all birds exhibit the typical spotty liver lesions, an observation that was also supported by others (13). In challenge studies of *C. hepaticus* inoculated layers at a concentration of 10^9 to 10^{10} cfu/bird, lesions were detected in nearly every bird and they appear to shed the organism in feces (15) which may account for the horizontal spread of SLD. Researchers have noted that *C. hepaticus* may cause enteritis (12), can rapidly move from the intestines to the liver (9), and appears to be more invasive than other *Campylobacter* species. The organism is bile resistant (16) and has been found at higher levels in bile than in the liver (15). Despite these studies there is, however, limited information on the pathogenesis of *C. hepaticus* and genome sequencing of one strain noted there was a reduced number of pathogenicity genes (17).

Currently, there are no approved treatments for SLD in organic flocks, however companies may respond by acidifying the water with products such as apple cider vinegar or citric acid, and/or treating with a non-antibiotic intervention such as oregano. In severe cases, an antimicrobial such as chlorotetracycline may be used in the feed (trade name Aureomycin) with zero-day egg withdrawal if birds are being raised conventionally. Novel treatments such as isoquinoline alkaloids have been shown to induce partial protection of laying hens affected by SLD (18). In addition, biochar (a carbon substance) fed to chickens can lower the load of *C. hepaticus* and may decrease the incidence of SLD in layer hens (19). Currently, there is no research elucidating the effect of treating hens with acidifiers in the water. Also, to the author's knowledge, no other US group has been able to replicate SLD in table egg layers. Therefore, the objective of this study was to develop a challenge model for *C. hepaticus*. Analysis of clinical signs, gross lesions scores of the liver, mortality, and transmission of *C. hepaticus* to naïve exposed chickens were assessed. *C. hepaticus* presence was determined using bacteriology, PCR, and histopathologic scores.

MATERIALS AND METHODS

Bacteriology. Bacterial culturing was performed from bile samples collected from the gall bladder. All samples were logged and assigned an ID. For bile samples, a 30µL volume of the bile was plated directly on duplicate blood agar (TSA with sheep blood; Remel) plates and streaked out with a 10uL loop, allowed to soak into the agar and incubated at 37°C and 42°C under microaerophilic conditions for 7d. Plates were checked at 3 and 7d for suspect growth. Following incubation, all plates were inspected for colonies with typical *Campylobacter* morphology and suspect isolates were selected for PCR analysis.

Challenge bacteria. The *C. hepaticus* strain RBCL71delta (CP104325) along with three other isolates (RBCL76delta JAOBQM000000000; RBCL81delta JAOBQL000000000; RBCL91delta JAOBQK00000000) from Georgia, US were used to challenge birds. These strains were isolated from an organic pasture raised layer operation showing gross lesions later confirmed as SLD via culture and PCR (10). The bacteria were cultured on blood agar and colonies were picked into phosphate buffered saline (PBS) and all 4 strains were mixed equally to generate an inoculum dose of 10^7 cfu/mL/bird. Birds were inoculated orally three times during the trial with the same challenge dose at days 1, 4 and 7.

Experimental design. A total of 43 SPF chickens, 30 hens and 5 rosters, were raised together in floor pens sharing the same cage free environment, with no outside access. All bird studies were carried out under IACUC approval (A2022 03-014-A1). At 17 weeks of age, birds were randomly divided into 2 groups; 18 birds from the positive control groups (n=35) were orally challenged with the *C. hepaticus* cocktail (RBCL71delta; RBCL76delta; RBCL81delta; RBCL91delta) and 1 negative control group (n=8, 4 hens & 4 rosters). Only 51% (n=18) of the population of the positive control group were orally challenged with the *C. hepaticus* RBCL cocktail. Groups were placed in 2 separate colony houses with a minimum of 1- 2 feet² of floor space/bird and all were tagged with an ID number. Before challenge, 3 additional random non-challenged birds were selected and euthanized to collect bile, liver tissue, and observe for the presence of SLD gross lesions to confirm that birds were free of *C. hepaticus*. On day 9 of the trial, 6 orally challenged hens and 5 non-challenged hens were euthanized to collect samples for bacterial

culture and to record gross lesions. On day 15 post challenge, five of the challenged birds and five naïve non-challenged birds per group were euthanized, and samples were collected for culture, and gross lesions were recorded. On day 20 post challenge, 5 challenged chickens and four naïve non-challenge birds were euthanized for sample collection. At day 27 post challenge, all remaining birds were euthanized, tissues were collected for culture, and gross lesions were recorded (see Figure 1). During the entire duration of the project, clinical signs, mortality, feed intake, and water intake were recorded. All birds had ad libitum access to water and feed.

Clinical signs, gross lesions, and mortality. Clinical signs were based on lethargy, diarrhea, and feed refusal/decreased feed intake. Gross liver lesions scores were calculated for each bird. Briefly, the livers were scored on a scale of 0-3 based on gross lesions characterized by multifocal, small, round, white foci on the liver surface. A score of (0) indicated no lesions observed, mild (1) < 20 spots), moderate (2) > 20, but < 60 spots), and severe (3) > 60 spots or too numerous to count). Mortality or any bird that was euthanized due severe *C. hepaticus* clinical signs or due to criteria set out in the IACUC protocol received a score of 3.

DNA extraction. Suspect isolates of *Campylobacter* recovered from bile samples were picked and added to 100µL of sterile water in a 200µL tube. Then DNA was extracted using the boil prep method. DNA extraction from liver tissue was performed using the protocol for DNA Purification from Tissues Using the DNEasy Power Soil Pro Kit (Qiagen, Germany).

Polymerase chain rection (PCR). Amplification of the target DNA for *C. hepaticus* detection was carried out using primers targeting the glycerol kinase gene, using primers G2F3 and G2R2 (15).

RESULTS

Detection of *Campylobacter hepaticus* via bacteriology and PCR to assess infection of SPF chickens. The three birds euthanized before bacterial challenge were negative for *C. hepaticus* via bile culture and PCR of bile and liver with *C. hepaticus* specific primers and probes. Bile samples were collected at 9,15, 20, and 27 days post-challenge. Due to the low number of birds, statistical analysis was not performed. Results are expressed as a percentage of the total number of samples at 0 to 27 days post-challenge and the bile was the only sample used for bacteriological analysis.

The positive control group had 5/18 samples harboring *C. hepaticus* on bacteriological analysis of the bile and 5/18 samples were positive by colony PCR. As expected, the negative control group had no growth of *C. hepaticus* on any of the 8 samples tested and all were colony PCR negative (Table 1).

Clinical signs post challenge, survival, and gross liver lesions of challenge chickens. During the length of the experiment, birds were checked twice daily for any clinical signs and mortality. There were no clinical signs in any of the groups and no mortality was recorded. SLD gross lesions were observed in the positive control group only. The positive control group had 4/18 birds showing mild lesions resembling SLD, (Table 2 & Figure 1B). In addition, the positive control had 1/18 birds with moderate SLD lesions (Table 2 & figure 1C).

Detection of *C. hepaticus* in the liver post challenge. Liver samples collected at 9, 15, 20 and 27 days post challenge were analyzed via direct PCR of extracted DNA. Results are expressed as total samples at 0 to 27 days post challenge. On direct PCR for *C. hepaticus*, the positive control groups had 18/18 positive on direct PCR (Table 1).

***C. hepaticus* challenge, clinical signs, and gross lesions for naïve contact chickens.** Naïve contact chickens were introduced on the same day as the challenge birds. From day 0 to 27 post-introduction, there were no clinical signs or mortality in any of the naïve contact exposure chickens. *C. hepaticus* culture from bile from day 0-27 post-introduction found that 2/17 naive birds in the positive control group were also positive (Table 1). Direct PCR from liver on the positive control birds showed that 15/17 birds were positive on liver PCR. Results showed that the positive group had SLD gross lesions on the naïve contact exposed chickens, in which 2/17 birds showed severe spotty liver lesions (Table 2 and Figure 1D).

DISCUSSION

The objective of this study was to develop a challenge model for *C. hepaticus*. To the authors' knowledge, this is the first reported study where spotty liver lesions have been reproduced in challenged birds in the US. Spotty liver disease appears to affect hens at peak production and during hot and wet weather seasons; although, in this study, *C. hepaticus* did not cause clinical signs after challenging birds at 17 weeks of age, but SLD lesions were seen post challenge. In an epidemiological study carried out in 2020, Phung et al. found that *C. hepaticus* can infect hens during the rearing phase and before they start to lay eggs with outbreaks of SLD occurring primarily during peak production (28 to 32 weeks of age), but SLD can also occur before or after the production cycle (8). In the current study, SLD was reproduced in chickens from 17 weeks of age onwards. Also, in this study, challenge birds had gross lesions

resembling those attributed to SLD. During the entire length of the study, all chickens appeared healthy and were consuming ~90 g of feed/ bird and 160 mL of water/bird. No significant differences in feed and/or water consumption were observed among groups following challenge. On necropsy, 4/18 challenged birds in the positive group had mild spotty liver lesions and one bird had moderate spotty liver lesions. Naïve non-challenged birds in the positive group, 2/17 birds had severe SLD lesions. In addition, liver lesions appeared to decrease or disappear within a couple of days of infection with *C. hepaticus* (9). There is no effective treatment for *C. hepaticus* or commercially available vaccine to control SLD. Novel treatments such as isoquinoline alkaloids and biochar have demonstrated to reduce SLD in layer hens (18,19). It is not well understood how fast infected hens with *C. hepaticus* die as clinical signs are often missed because the infected hens often die quickly. In this study, mortality before and after treatments was not observed in any of the treatment groups, even with the high *C. hepaticus* dose (10^7cfu) given 3 times over a period of 5 days. Nevertheless, the birds used in this study were housed under extremely comfortable conditions with lower stocking density and limited stress which may not be reflective of actual industry conditions.

During this study, agar plates that had colonies characteristic of *Campylobacter* were confirmed with PCR. It is essential to know that some *Campylobacter* species cannot be differentiated using culture plates alone; therefore, PCR must be run to confirm *C. hepaticus* in a culture and to differentiate it from other *Campylobacter* species. It has also been shown that *C. hepaticus* can enter a viable but non-culturable state during stress, which can prolong the survival of *C. hepaticus* in the environment and makes it difficult to isolate in culture from environmental samples (20). In this study, a number of collected bile samples did not show growth on culture; however, many of those negative culture samples were positive for *C. hepaticus* on liver analysis and PCR from positive control group. This observation suggests that *C. hepaticus* in bile can enter a viable but non-culturable state and therefore may not be easy to culture. Also due to the slow growth rate of *C. hepaticus* on culture media, other bacteria can grow and outcompete it making it difficult to identify *C. hepaticus* on culture or colony PCR due to the high number of other non *C. hepaticus* bacteria contaminating the plates.

Typically, free-range flocks are moved to the hen house between 15 to 17 weeks of age. They are allowed to free range outside the house after 25 weeks of age, and peak production can start as early as 26 to 30 weeks of age. SLD is observed primarily in free-range hens that have access to the outdoors and are at peak production, which could indicate that a few hens can become infected as soon as they have access to free range outside and can bring the organism back to the house where it can spread to other birds. In this study, the theory of fecal-oral transmission for *C. hepaticus* is supported as challenged birds became infected and were able to horizontally transmit the organism to naïve non-challenged birds. Previously, Becerra et al., 2023 showed that feces was a potential source of contamination in the barn and persisted for more than 10 weeks despite ongoing treatment with apple cider vinegar and oregano (5). Such data suggests that controlling *C. hepaticus* once established will continue to be a challenge.

It is not well known what infectious dose of C. *hepaticus* is required to cause disease in hens. Van et al., was able to infect 26-week-old hens and show gross lesions resembling SLD with doses of $1x10^9$ and $1x10^{10}$ cfu of *C. hepaticus* (9). In the present study, chickens were infected with $1x10^7$ cfu on three different occasions, which was meant to be reflective of repeated exposure events that probably occur in field settings. It was expected the challenge dose would result in some mortality and that many chickens would develop severe SLD lesions; however, there were no clinical signs, or mortality, and most of the SLD lesions were mild. The findings from our current study suggest that the dose to see gross lesions and clinical signs, attributed to SLD could be extremely high. Further work to assess the pathophysiology and the endemic status of *C. hepaticus* in flocks is warranted. Since there are no approved treatments or vaccines currently available for *C. hepaticus*, further research is necessary to identify effective treatment and prevention strategies for SLD in hens.

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Figure 1. Livers showing gross SLD lesions. (A). A score of (0) indicated regular, (B). mild (1, less than 20 gross spots in the liver), (C). moderate (2, more than 20, but less than 60 spot in the liver), and (D). severe (3, over 60 spots in the liver or too many to count).

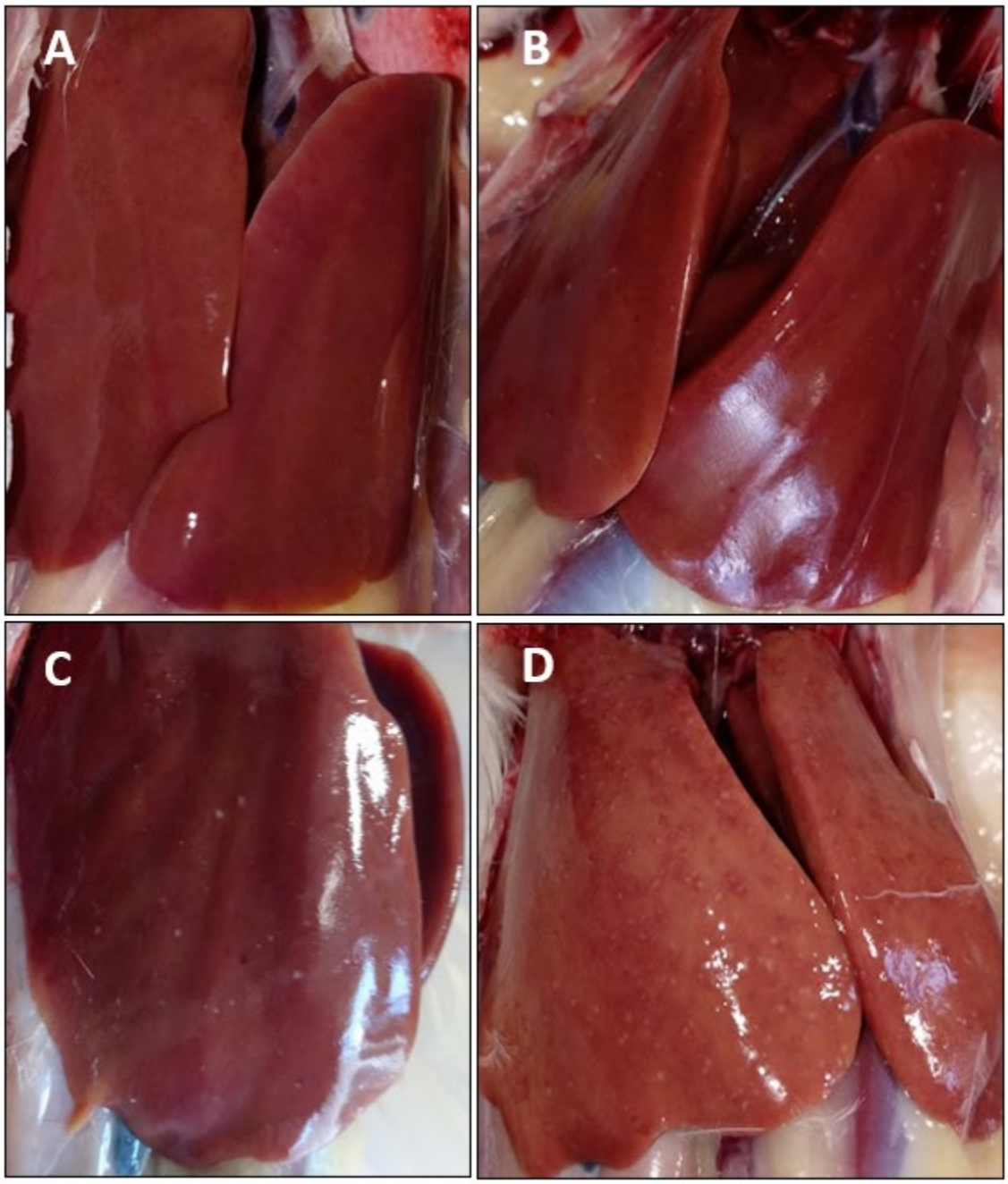

Table 1. Detection of *C. hepaticus* in challenged birds and naïve exposed non-challenged birds.						
	PCR positive/total					
Timeline	0 to 27d post-challenge			0 to 27d post-introduction of naïve birds		
Treatment & sample type	*Campylobacter* morphology	Colony PCR	Direct PCR	*Campylobacter* morphology	Colony PCR	Direct PCR
Positive control						
Bile	5/18	6/18	NT	2/17	3/17	NT
Liver	NT	NT	18/18	NT	NT	15/17
Negative control						
Bile	0/8	0/8	NT	NT	NT	NT
Liver	NT	NT	0/8	NT	NT	NT

NT = not tested

Table 2. The presence of SLD lesions in challenged and non-challenged bids						
	SLD lesions/total					
Treatment	Challenged birds			Naïve non-challenged birds		
	Mild lesions	Moderate lesions	Severe lesions	Mild lesions	Moderate lesions	Severe lesions
Positive control	4/18	1/18	0/18	0/17	0/17	2/17
Negative control	0/8	0/8	0/8	NT	NT	NT

NT = not tested

AVIAN REOVIRUS SURVEILLANCE IN MEAT-TYPE CHICKENS FROM THE CALIFORNIA CENTRAL VALLEY BETWEEN 2019 AND 2022

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SUMMARY

Avian reovirus (ARV) causes pathologies in meat-type birds especially chickens and turkeys. Due to its RNA nature and segmented genome, the virus is subject to mutations and genetic recombinations that lead to the emergence of new variants. This study focuses on the molecular characterization of ARV strains from different field outbreaks occurring in one meat type chicken producer in the State of CA between 2019 and 2022. Of 40 isolates, 11 were successfully sequenced, and phylogeny was performed using reference strains as a backbone. The studied isolates distributed in different genotypic clusters than the conventional vaccine strains. This surveillance study contributes to the understanding of the epidemiology of avian reoviruses in California.

INTRODUCTION

Avian reovirus (ARV) is responsible for causing pathology in meat-type chickens and turkeys (10). It is a non-enveloped virus containing a double stranded RNA genome comprised of 10 segments. These segments vary in their sizes and are classified into 3 categories: 3 large (L1, L2, L3), 3 medium (M1, M2, M3), and 4 small (S1, S2, S3, and S4) (3,5). Among these segments, S1 encodes for sigma C protein which is part of the viral capsid and is involved in host cell attachment and elicits neutralizing antibodies (5,8). Being a hypervariable protein, sigma C serves as a genetic marker in classifying different ARV genotypes and is well characterized in broilers (1,11,13,14). Due to the segmented RNA viral genome, the virus is prone to mutations and genetic recombination leading to the emergence of new variants (4–6). Six distinct genotypic clusters were identified according to the classification proposed by Kant and Lu (5,9,11) but a 7th genotypic cluster was reported in 2019 (16). In broilers, ARV infection is characterized by respiratory and enteric diseases, runting-stunting syndrome, hepatitis, and myocarditis (3,6). But the most prominent feature of this virus is viral arthritis and tenosynovitis causing hock joint swelling and lesions in the gastrocnemius tendon and its sheath. Though ARV variants have been reported in US since 2011 (2,11,15), the surveillance of ARV started in California in 2015. Poultry flocks have been historically immunized against the virus with commercial modified live and inactivated vaccines including classical strains, S1133, 1733 and 2408. However, these vaccines fail to protect the birds against the field challenge composed of novel ARV variants, reported in different outbreaks around the world. This suggests that variants bypass the immunity induced by conventional vaccine strains (5,7,12,18). This study describes the isolation of ARV strains from 2019 to 2022 and molecular characterization of ARV isolates based on partial S1 gene sequences. This surveillance and characterization can be utilized to observe the variations in ARV strains in the field and provide guidance in selecting the candidate for autogenous vaccine formulation.

MATERIALS AND METHODS

ARV isolation. Tissues (heart, liver, joint fluid and tendons) were collected from tenosynovitis suspected cases in broiler chickens between 2019 and 2022 in California. Virus isolation was performed using chicken embryo liver (CEL) cells (5). Samples showing no detectable cytopathic effects (CPEs) after five days were subject to a second passage. Confirmatory RT-qPCR was performed to detect the presence of the ARV genome (17).

Molecular characterization. A 1088 bp segment of the S1 gene was amplified by RT-PCR and molecularly characterized. Oligonucleotides used for this amplification were previously described (9). PCR products of positive

samples were purified using QIAquick® PCR Purification Kit according to the manufacturer's protocol, and dsDNA concentration was determined using nanodrop in each sample. The purified PCR products were sent for Sanger sequencing. Adequate sequences were obtained from 11/40 isolates. These sequences were aligned with 123 previously published sequences along with three commercial vaccine sequences, S1133, 1733, and 2408 using Geneious Prime® (Java Version 11.0.15+10). Phylogenetic analysis was performed using RaxML method with 1000 bootstrap replicates.

RESULTS

ARV isolation. Forty reoviruses were effectively isolated from the collected samples. All isolates were confirmed as positive for avian reovirus using the previously described RT-qPCR.

Molecular characterization. Of 40 isolates, 11 sequences of a portion of the S1 gene were obtained. Phylogenetic analysis grouped the isolates into six distinct genotypic clusters (Figure 1).

DISCUSSION

In this study, 40 reoviruses were detected and isolated between 2019 and 2022. Eleven isolates were successfully amplified and sequenced. The lack of amplification in about 73% of the isolates might be attributed to failed sequencing or poor quality and/or quantity of the extracted DNA. Our data showed that the studied ARV variants distributed in different genotypic clusters (9). Previous classifications showed ARV's distributing in all six genotypic clusters and the most prevalent were GC1 and GC6 with 51.8% and 24.7%, respectively (5). Our current results show a shift in this trend with isolates distributed in GC2, 3, 4 and 5, and no isolate grouped in GC1 or GC6. Most of the isolates grouped in GC2 and GC5 (36.4% each). Our surveillance effort has demonstrated the shifting of ARV isolates (5,14). This study will help in investigating a long-term picture of reovirus epidemiology in California and may guide us to strategize alterations in the vaccine schedule for the disease control.

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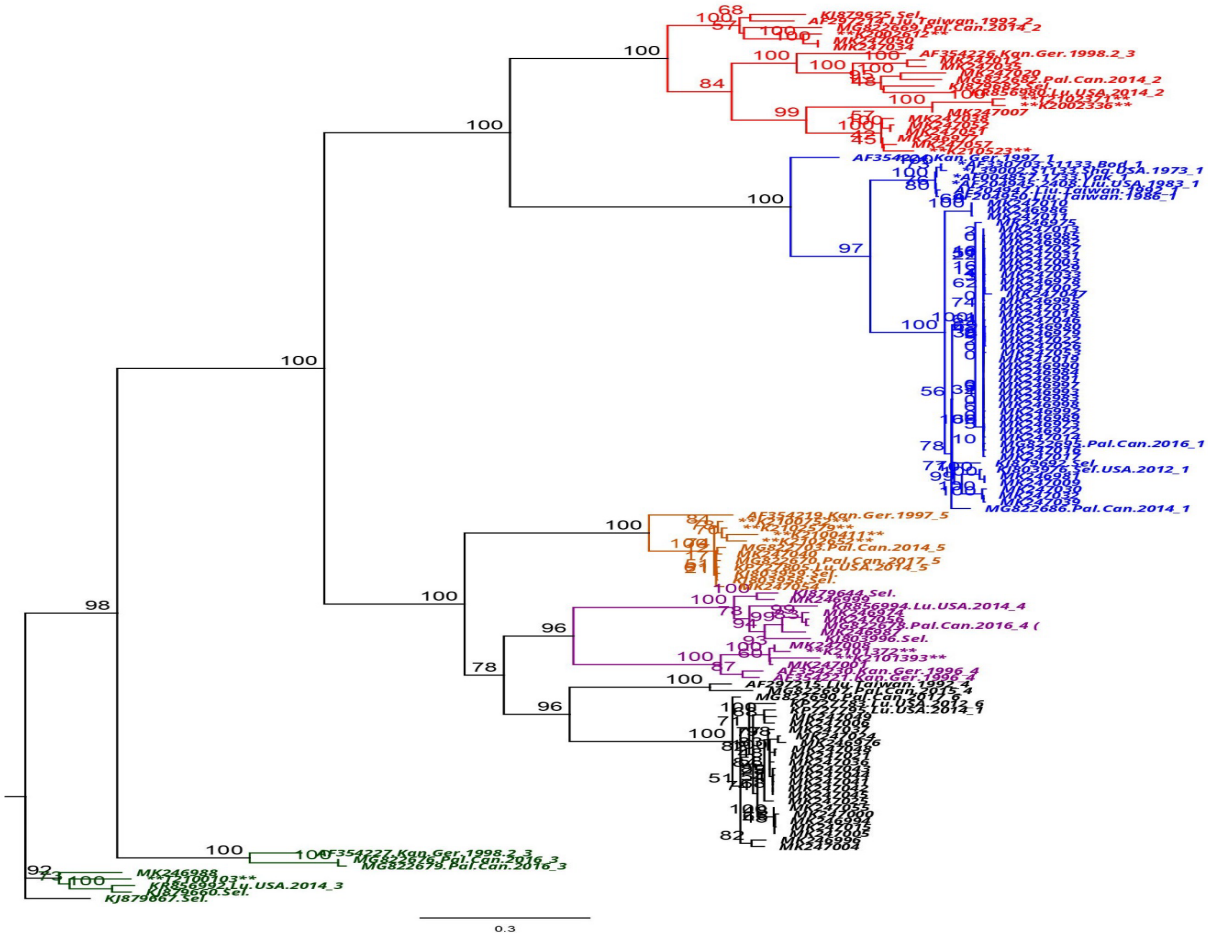
Table 1. Sequences by genotypic cluster (GC) between year 2019 and 2022 with percentage.
ND=Not Detected

Genotypic cluster (GC)	Sequences from year 2019 to 2022	Percentage (%)
GC1	ND	0
GC2	K2002612 T2102371 K2002336 K210523	36.4
GC3	T2100103	9.1
GC4	K2101372 K2101393	18.1
GC5	K2100752 K2102579 K2100411 K2102655	36.4
GC6	ND	0

Figure 1. Maximum likelihood phylogenetic tree showing 137 partial S1 gene sequences with 1000 bootstrap values. The vaccine sequences were labelled by a single asterisk (*) and the 11 obtained sequences were bold, italicized and highlighted using double asterisks (**). Genotypic cluster (GC) 1 was colored blue, GC2 red, GC3 green, GC4 purple, GC5 orange and finally GC6 was black. The study sequences were represented in Italic, bold and had double asterisks (**).



INTERPRETATION OF OPGS: MISSION IMPOSSIBLE

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Oocysts per gram (OPGs) are used to evaluate anticoccidial efficacy and immunity development. Interpretation is complicated by: Type of coccidiosis prevention, age of the flock, stocking density, initial level of contamination, type and amount of sample collected, downtime period, number, and location of samples, collected, bird's immune status, crowding effect, predominant Eimeria spp., storage conditions and time of storage, and others. Experimentally, OPGs are much more useful because the influence from most of the factors listed above plus the many uncontrolled variables from the field are minimized. As more poultry is produced in NAE, RWA, and organic programs, the use of synthetic anticoccidials and vaccines has increased. Development of resistance to synthetic anticoccidials can be problematic and OPGs could be used as an early warning sign. OPGs can also be used to monitor proper cycling of coccidial oocysts and development of active immunity in birds vaccinated with live coccidiosis vaccines.

STUDIES DEMONSTRATING THE POTENTIAL TO ENHANCE IMMUNITY AND IMPROVE OVERALL EFFICACY OF LIVE *E. COLI* VACCINATION BY PARENTERAL ADMINISTRATION

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INTRODUCTION

When the live poultry vaccine for *E. coli* (PVEC) was introduced almost two decades ago, it was instrumental in helping to dramatically reduce mortality losses from *E. coli* peritonitis in laying birds. Since then, several challenge studies have demonstrated its utility in significantly reducing the incidence and severity of disease and percent mortality against various serotypes and pathotypes—even well into lay. However, with the constant evolution of our industry (the bird, the bug, new production systems, other emerging primary and secondary diseases, etc.), *E. coli* peritonitis mortality has become one of today's top threats to achieving optimal hen production. This paper summarizes our first two studies conducted to explore the potential that higher levels of protection might be achieved by adding an injection of the PVEC into pullet programs.

MATERIALS AND METHODS

Six-week-old SPF leghorns were placed on fresh litter at the Animal Research Center (ARC). In Study 1 (see table), half the birds were vaccinated with PVEC by eye drop upon arrival while the other half remained not yet vaccinated. At 11 weeks, the two treatments were each subdivided and either non vaccinated, eye drop vaccinated or intramuscular (IM) injected. In Study 2, at 10 weeks of age all birds received a killed poultry vaccine for *Salmonella* Enteritidis (PVSE) and were subdivided by PVEC vaccination status: 1) none, 2) eye drop, 3) IM (opposite breast as PVSE) or 4) IM (mixed with PVSE and injected IM same breast. In both studies, birds from each treatment were commingled and challenged intratracheally at 17 weeks with an avian pathogenic *E. coli* (APEC) using 10^9 or 10^10 organisms per dose (see tables). Study 1 and Study 2 (10^10 dose) used 25 birds per treatment and Study 2 (10^9 dose) used 40 birds per treatment. Birds were weighed at time of challenge and a week later at termination of study when lesions were scored. All hypotheses were conducted at the $p \leq 0.05$ level of significance with the Shaffer simulated method used to adjust for multiple comparisons.

RESULTS

In Study 1, the never vaccinated control treatment T01 had the highest rate of mortality (30%) followed closely by the once-vaccinated eye drop groups at 11 weeks (29%) and 6 weeks (21%). The twice vaccinated eye drop group was fully protected from mortality as were the IM vaccinated groups—with or without an eye drop primer vaccination. The one-time eye drop treatment vaccinated at 11 weeks had a similar rate of diseased birds (54%) as the non-vaccinated controls (57%). However, the one-time eye drop treatment vaccinated at 6 weeks had intermediate levels of colibacillosis (33%) similar to the two-time eye drop vaccinates (29%) or unprimed IM vaccinates (36%). Finally, the IM group that was first primed with an eye drop vaccination had the lowest incidence of colibacillosis (16%) in the study.

In Study 2, low-level mortality was seen in the non-vaccinated challenge controls as well as the eye drop vaccinates receiving the higher 10^10 colony forming unit (CFU) dose. All other treatments had no mortality. The 10^10 CFU challenge dose consistently yielded higher rates of lesions and weight suppression across all vaccine treatments. Within each lesion category, the order of increasing efficacy by vaccination was eye drop to IM (separate breast injection from PVSE) to IM (mixed with PVSE and given in same breast). Eye drop vaccination significantly reduced colibacillosis against the 10^9 CFU dose but not against the higher 10^10 CFU dose. PVEC given IM by itself (T03) significantly reduced airsac and colibacillosis incidence against both challenge levels. However, when

PVEC was mixed with PVSE and combined in one breast injection it lowered the rate of colibacillosis significantly yet against the 10^9 CFU dose compared to when PVEC was injected by itself.

DISCUSSION

Study 1 demonstrated some subtleties in efficacy based on the timing and route of PVEC application. The one-time eye drop treatments did not prevent significant mortality but the 6-week application performed numerically better than the 11-week application even though the interval between vaccination and challenge was almost twice as long. The two-time eye drop treatment did prevent mortality, however, showing the value of a primer/booster program. The unprimed IM treatment prevented mortality unlike the analogous eye drop treatment—showing a fundamental difference in immunity when delivering PVEC parenterally even without a primer. That said, the IM group that received a previous eye drop vaccination resulted in the lowest rate of colibacillosis—showing there is a value to a primer/booster strategy even when vaccinating IM.

Study 2 did not involve a primer/booster strategy—just a one-time PVEC application. However, all treatments did receive a full dose of killed salmonella PVSE to see if PVEC would perform even better if mixed with PVSE and injected. The eye drop treatment prevented mortality and significantly lowered colibacillosis incidence against the 10^9 challenge but not the 10^10 challenge—suggesting that even a one-time eye drop treatment can reduce disease from APEC as long as the challenge is not too heavy. The one-time IM treatment was clearly superior to eye drop and the difference was most apparent against the higher 10^10 challenge level. Finally, IM PVEC performed consistently the best when it was mixed with the killed *Salmonella* PVSE before injection. This suggests that there is an additional benefit to presenting the live PVEC with the adjuvanted vaccine.

In conclusion, these studies show the potential to attain even higher levels of PVEC immunity by adding an injection application. This may be especially valuable when raising birds under more stressful and higher challenge conditions.

Study 1. *E. coli* vaccination by different times and routes and lesions from APEC (10^9 CFU) challenge at 17 weeks

Treatment	PVEC vaccination		Percent Mortality	Percent Colibacillosis
	6 weeks (Primer)	11 weeks		
T01	None	None	30a	57
T02		Eye Drop	29a	54
T03		Intramuscular	0b	36
T04	Eye Drop (primed)	None	21ab	33
T05		Eye Drop	0b	29
T06		Intramuscular	0b	16*

*Treatment 6 is nearly significantly lower than treatments 1 and 2 (P=0.0652).

Study 2. Results by different *E. coli* vaccination techniques after APEC (10^9 or 10^10 CFU) challenge at 17 weeks

PVEC Treatments (10-week vaccination)		%Mortality		% Airsac		%Colibacillosis		Weight Gain (g)	
		10^9	10^10	10^9	10^10	10^9	10^10	10^9	10^10
T01	None	3	4	47a	73a	69a	78a	-98a	-147a
T02	Eye Drop	0	8	32ab	59a	40b	83a	-71a	-100a
T03	Intramuscular	0	0	15bc	24b	33b	36b	-25ab	-31b
T04	Intramuscular (with PVSE)	0	0	5c	16b	10c	24b	+6b	-2b

STUDIES DEMONSTRATING THE POTENTIAL TO ENHANCE IMMUNITY AND IMPROVE OVERALL EFFICACY OF LIVE *SALMONELLA* TYPHIMURIUM (ST) VACCINATION BY PARENTERAL ADMINISTRATION

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INTRODUCTION

Live ST vaccines have been in use for over two decades to help control salmonella. A typical vaccination program includes 2-3 live ST vaccinations in the first several weeks of the pullet's life followed by 1-2 inactivated vaccinations prior to the point of lay. One common perception is that live salmonella vaccines only induce a limited duration of immunity and little, if any, humoral antibody response1. Currently, live vaccines are only being applied by mucosal application—coarse spray or drinking water. However, because of the unique properties of the aro-A attenuated live ST vaccine2, recent studies have been designed to see if this vaccine might provide additional benefits not currently being realized in today's vaccination programs. This paper will summarize three different studies—one exploring the efficacy of injecting live ST at day of hatch and two others where the ST vaccine is injected with or without a killed *Salmonella* Enteritidis (SE) bacterin at 10-12 weeks of age.

MATERIALS AND METHODS

SPF leghorns were either hatched at the Animal Research Center (ARC) for the day of age vaccination study or arrived there as 6-week-old pullets. Treatment groups were raised on fresh litter in separate pens after vaccination but commingled at the time of challenge. In the day of age vaccination study, the conventional (mucosal) group received live ST at day of age and then again at 14 days. Each group was bled for Group B Salmonella ELISA titers at 30 days, orally gavaged with 10^9 colony forming units (CFU) SE at 32 days and cecae and spleens were harvested at 43 days. In the injection studies in older birds, Study 1 birds were vaccinated at 12 weeks (see Table 2 for treatments), bled at 19 weeks, challenged with 10^8 CFU S. Heidelberg (SH) at 20 weeks and cecae and spleens were cultured 11 days later. Study 2 birds were vaccinated at 10 weeks, bled at 16 weeks, challenged at 17 weeks with 10^9 CFU SE and sampled 10 days later. For all three studies, 15 birds were bled and 40 were challenged in each treatment. Cecae, spleen and ovarian follicles were collected for Most Probable Number (MPN) enumeration3 and enrichment for prevalence, if negative. Super shedders were statistically determined by applying cluster analysis to MPN results. All hypotheses were conducted at the $p \leq 0.05$ level of significance with the Shaffer simulated method used to adjust for multiple comparisons.

RESULTS

In the day of age vaccination study, both live ST vaccination treatments showed fewer positive spleens, lower loads in cecas and spleens and fewer super shedders in cecas and spleens. Cecal loads and % super shedders were lowest in the conventional mucosal application treatment while spleen % positives and super shedders were lowest and loads were significantly lowest in the subcutaneous application treatment. Group B serology is pending.

In the long-lived challenge study (SH), injection of the live ST vaccine resulted in a serum response on the Group B ELISA test (1830). The ELISA response was 30% higher to the killed SE injection but the combination of live/killed vaccines resulted in another 60% increase over the killed SE. All three vaccine treatments resulted in significant reductions in ceca and spleen loads. Live and Live/killed treatments showed lower spleen positives and loads than Killed SE but these differences were not significant.

In the long-lived challenge study (SE), there was not a live ST-only treatment but the addition of live ST to the Killed SE vaccination resulted in ~50% increase in the Group B ELISA test. Both vaccine treatments resulted in

significant reductions in cecal, splenic and ovarian follicle loads. The addition of live ST to the Killed SE resulted in lower cecal and ovarian loads over Killed alone and significantly lower prevalence and loads in spleens.

DISCUSSION

These studies demonstrate that the application of a live ST vaccine by injection has the potential to provide a different type of immune response compared to oculonasal (spray) or oral delivery. The first obvious difference is this aro-A attenuated live ST vaccine elicits a serological response by injection that it doesn't by mucosal application1—perhaps due to the exposure of much more vaccine to circulating mononuclear cells. It is interesting that the injection response—while respectable on cecal protection—seems to be even more beneficial to internal organ protection. It should also be noted that a single injection resulted in significant protection for at least 8 weeks without the benefit of previous priming—something that mucosal delivery of live ST vaccine has not demonstrated. Finally, while Killed SE vaccine (Group D) demonstrated cross reactivity on Group B ELISA and resulted in significant reductions in SH and SE loads (up to 2.5 logs), the co-injection of live and killed vaccines resulted in even fewer positive organs and greater load reductions (up to 4 logs). While vaccination has been instrumental to food safety risk reduction programs in poultry, the vaccine options have not changed much in the past few years. The three studies presented here show the potential of improving salmonella vaccine protection simply by working differently with what we already have.

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Table 1. Conventional ST vaccination vs. subcutaneous day of age injection study comparison against SE challenge*

Vaccination Group	% Prevalence		% Super shedders		Loads (GMT)	
	Cecas	Spleens	Cecas	Spleens	Cecas	Spleens
No live ST	100	92	47	55	3163	392a
Mucosal D1+14	100	85	33	40	579	166a
Subcutaneous D1	100	70	35	3	1077	3b

*SE challenge by oral gavage at 10^8 CFU/bird at 32 days of age and cultured 11 days later.

Table 2. Comparing SE and/or ST vaccination by injection at 10-12 weeks of age and challenged 7-8 weeks later.

Vaccination Group	Group B ELISA GMT*		% Positive Spleens		Cecal loads (log10 GMT)		Spleen loads (log10 GMT)		Ovarian follicles (log10 GMT)
	Study 1	Study 2	SH**	SE***	SH	SE	SH	SE	SE
No vaccine	74	184	100a	85a	4.9a	2.9a	3.1a	4.2a	1.5a
Killed SE	2400	3089	50b	65a	1.8b	1.4b	1.0b	1.5b	0.4b
Live ST	1830	--	42b	--	1.6b	--	0.3b	--	--
Killed SE+ Live ST	3808	4600	36b	18b	2.0b	1.2b	0.7b	0.0c	0.1b

*Serology was measured 1 week prior to challenge.
**Study 1 = SH challenge and ***Study 2 = SE challenge—both by oral gavage at 10^9 CFU/bird.

HIGHLY PATHOGENIC AVIAN INFLUENZA OUTBREAK IN A BARN, WHAT TO EXPECT

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ABSTRACT

The current HPAI outbreak is challenging the poultry industry biosecurity measures. In addition, migrating birds pose a constant menace to poultry owners. We collected environmental and oropharyngeal swabs in an infected turkey premise for four days after HPAI was diagnosed, the idea was to track viral decay and / or persistence. Viral load was assessed by RT-qPCR and virus viability by embryo inoculation. Higher viral loads by RT-qPCR were detected in birds either composted or non-composted. When these samples were assessed for viral viability, we found live virus in composted and non-composted birds until 3 days after euthanasia. In the environment live virus was only found 1 day after depopulation. These results raise the concern for extended down time periods in poultry premises after HPAI outbreaks.

INTRODUCTION

Biosecurity is key to avoid the introduction of pathogens into poultry farms, but when we have ranches with active outbreaks, biosecurity becomes key to prevent dissemination of infectious diseases. The current HPAI outbreak is challenging biosecurity measures. Migrating birds pose a constant menace to the industry and the industry has responded with good biosecurity. Proof of that, is that most of the affected farms in the US have been linked to single introductions and not with biosecurity breaches between farms (1). Despite this, outbreaks keep happening jeopardizing the food chain and stability of the business. One important aspect of containment is to understand what happens with the virus inside infected premises. For that we collected environmental and oropharyngeal swabs in an infected turkey premise for four days after HPAI was diagnosed, the idea was to track viral decay and / or persistence. Viral load was assessed by RT-qPCR and virus viability by embryo inoculation.

MATERIAL AND METHODS

Swabs were collected from the environment, i.e., drinkers, trash bins, house dust, at different locations of the affected barn and birds either composted or non-composted for a period of 4 days after HPAI RT-PCR confirmation. These swabs were stored on ice packs and delivered to the California Animal Health and Food Safety Laboratory (CAHFS) for RT-PCR (2) and embryonated egg inoculation for virus viability assessments (3)

RESULTS AND DISCUSSION

If we look at viral load obtained from RT-qPCR results, we can notice that oropharyngeal swabs from birds show higher viral loads than environmental swabs. These differences are statistically significant. No differences were detected between composted and non-composted bird swabs.

These results emphasize the importance of euthanizing birds rapidly after detecting the disease. It also shows that viral particles remain high in birds disregarding the compost treatment, reason why biocontainment during outbreak management is crucial. Since the above-mentioned results are detections by molecular techniques, they don't really give us an idea of the viability of the virus. For that, we performed embryonated egg inoculations of each sample and after that RT-qPCRs were performed. CT values were compared with the initial RT-qPCR to determine a reduction of the CT meaning that the virus replicated in the egg (live virus). Table 1 summarizes these results.

HPAI remains alive for up to three days in composted and non-composted birds, on the other hand the virus in the environment doesn't survive well getting inactivated before the first 24 hours. Previous results have shown that HPAI remains alive in bedding material and layer manure for up to 96 hours (3). When composting is used as a method

of virus inactivation, temperatures are critical on the effectiveness of inactivation of HPAI (4). In addition, we have seen that acidifiers have a positive effect in the inactivation of LPAI in spiked bedding material (4)

In conclusion, it is very important to act quickly in HPAI positive flocks, HPAI can remain alive in dead birds for up to 3 days, reason why biosecurity remains important even after depopulation. While the virus can be detected in the environment this virus is dead after three days reason why revisiting downtime of infected premises is important to privilege business continuity.

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Table 1. Summary table of the viability of HPAI in collected samples during the first 4 days after HPAI confirmation. Pos: live virus, Neg: dead virus, NT: not tested, CB: composted bird, B: non composted bird, Env: environmental swab.

	CB1	CB2	CB3	B1	B2	Drinkers S	Drinkers N	Env S	Env N
Day 0	Pos	Pos	Pos	Pos	Pos	Pos	NT	NT	NT
Day 1	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg
Day 2	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg
Day 3	Pos	Pos	NT	Pos	Pos	Neg	Neg	NT	Neg
Day 4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NT	Neg

VIABILITY OF AVIAN LIVE VIRUS AND BACTERIAL VACCINES AFTER PROLONGED STORAGE AT VARIOUS TEMPERATURES

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SUMMARY

Live vaccines made with selected vaccine strains of attenuated/modified viruses or bacteria are commonly used in the poultry industry worldwide. To ensure stability and efficacy of these vaccines, the lyophilized preparations are stored at 2-7°C. The normal expiration period for these vaccines varies from 1.5 to 4 years. Published literature on the viability of these vaccine strains after prolonged refrigeration at 2-7°C and beyond the expiration dates is scanty or unavailable. In this study, we evaluated the viability of live viral and bacterial vaccines after storage at refrigeration and lower temperature for a maximum period of up to 33 years. The results indicated that all lyophilized live virus and bacterial vaccines remained viable during the period of their long-term storage at varying temperatures. Although there was an expected loss of virus titer during storage, all of the vaccines tested still retained their infectivity and could be easily recovered by passaging in susceptible embryonated chicken eggs or in cell cultures. The only bacterial vaccine tested in this study was a lyophilized preparation of *Pasteurella multocida* (CU strain). This strain was also found to be viable after storage for 32 years.

INTRODUCTION

Vaccines require proper cold storage temperatures to prevent loss of product titer. A lyophilized vaccine when stored at the recommended refrigeration temperature (2-7°C) usually retains its efficacy during its expiration period assigned by the vaccine manufacturer. In order to maintain product integrity/potency, a cold chain storage system is implemented when transporting vaccines (3, 10). If kept at suboptimal conditions, vaccines quickly degrade and eventually lose its potency (4, 16). One way to prevent this is lyophilization (4, 13, 15,16). Lyophilized vaccines are manufactured and stored with an average shelf life of 1.5 to 4 years for optimal potency. Using vaccines past the expiration date is not advisable as the vaccine may lose its efficacy. Lyophilized vaccines are prepared by drying the vaccines under freezing conditions to enhance the shelf stability of the formulations (3, 4, 6, 7, 16). Over time vaccine degradation occurs through physical and chemical pathways including higher temperatures, pH changes, repeated freezing and thawing, and prolonged exposure to higher temperature and light (1, 2, 5, 8, 9, 12 -14). The presence of excess water or liquid components in the vaccine preparations specifically enhance these pathways and prompt hydrolytic reactions or deamidation (4, 14, 16, 17). In this study, we evaluated some lyophilized vaccines that were stored at various temperatures for 24- 33 years past the expiration date for any remaining viability. The findings in this study could be helpful in determining the potential for retention of old and rarely available vaccine strains that are believed to be outdated or unrevivable. These findings may also be helpful in designing ambient conditions for proper storage and shipping of the vaccines under different geoclimatic conditions affected by extreme change in weather or environmental temperatures.

MATERIALS AND METHODS

Viral vaccines. The viral vaccines used in this study included infectious bursal disease (IBD), Newcastle disease (ND), infectious laryngotracheitis (ILT), infectious bronchitis Connecticut strain (IBV- Conn), infectious bronchitis Mass 48 strain (IBV- M48), fowl pox (FP), and reovirus 1133 strain (Teno- 1133). All the vaccines were lyophilized live virus vaccines manufactured by different vaccine manufacturers. The vaccines were obtained from various commercial suppliers. These lyophilized vaccines were initially stored at the manufacturer's recommended temperatures (2-7°C). Past the marked expiration date, the vaccines were stored at various temperatures as shown in the storage history of the vaccines (Table 1).

Bacterial vaccines. One lyophilized bacterial vaccine *Pasteurella multocida* recommended for use in chicken was used in this study. The vaccine represented (CU) strain of *Pasteurella multocida* marketed under the trade name VI Clemcol- C.

Storage history of the vaccines. Detailed storage conditions of the vaccines are shown in Table 1. The vaccines used in this study were exposed to a long chain of temperature changes for varying periods of time extending up to 33 years. For several years, all vaccines were initially stored at refrigeration temperature (2-7oC). From there, they were eventually moved and exposed to room temperature for 2 days after which they were stored at alternating conditions of refrigeration and sub-zero temperatures for a varying period of time until tested for their remaining potency or viability.

Passage in SPF eggs. Specific pathogen free (SPF) eggs were used for passaging ILTV, IBV-Conn, IBV- M48, IBDV, NDV and Teno-1133. Ten day old embryonated eggs were used for all viruses except for Teno-1133, where six day old eggs were used. Prior to use, all vaccines were reconstituted with 5 mL tryptose phosphate broth (TPB). Eggs were inoculated with 0.2 mL of the reconstituted virus and observed for 7 days for any virus induced mortality and/or specific lesions caused by the inoculated viruses. All dead embryos as well as all surviving embryos after 7 days post- inoculation were examined for lesions specific to the inoculated viruses. Samples were collected at this time for other lab tests for assessment of virus titer and/or virus identity.

Passage in cell cultures. The IBD vaccine was tested for viability by passaging in chicken embryo fibroblast (CEF) cells culture. Prior to infecting the cells, the lyophilized vaccine was reconstituted using cell culture medium without serum. The confluent cell monolayer was infected with the reconstituted virus and observed for development of specific IBDV induced cytopathic effects (CPE). Positive CPE indicated presence of IBDV virus in the tested sample.

Testing for viability of the lyophilized *Pasteurella multocida* vaccine. The lyophilized *Pasteurella multocida* (PM) vaccine that was stored at various temperatures over 32 years (Table.1) was tested for viability by inoculating the reconstituted vaccine onto blood agar and trypticase soy agar (TSA) plates. Each plate was inoculated with 0.5 mL of the reconstituted vaccine and incubated in a humidified incubator set at 37°C for 24 hrs. The colonies were identified as Pasteurella by Gram staining and colonial morphology.

RESULTS AND DISCUSSION

The results are summarized in Table 2.

All viruses tested were found to have quantifiable titer upon revival. The NDV titer in egg showed signs of NDV infection through embryo mortality. The virus yielded a titer of 106.1 EID50/mL after 32 years past the expiration date. The revived NDV egg passaged virus (allantoic fluid) mixed with NDV specific antiserum showed no agglutination on hemagglutination inhibition test, confirming the virus identity to be NDV. The embryo passaged ILT vaccine displayed typical lesions indicative of the virus showing plaques on the chorioallantoic membrane (CAM). The virus yielded a titer of 103.5EID50/mL after 25 years past the expiration date. The FPV vaccine inoculated into the embryos also developed pock lesions throughout the (CAM). The virus yielded a titer of 106.2EID50/mL after 32 years past the expiration period. The Teno- 1133 vaccine caused embryo mortality and resulted in small embryos with purple discoloration. The virus yielded a titer of 107.3 EID50/mL after 24 years of storage. The IBV- Conn caused inoculated embryos to appear dwarfed and curled. The virus yielded a titer of 105.2 EID50/mL after 29 years of storage past the expiration date. The IBV- M48 vaccine also caused inoculated embryos to appear dwarfed and curled. The virus yielded a titer of 106.2 EID50/mL after 26 years in storage past the expiration date. IBDV was successfully passaged in cell culture after 33 years past expiration with visible CPE in the infected cells. The only bacterial vaccine (*Pasteurella multocida*) revived after 32 years showed bacterial growth on TSA and blood agar plates. The bacteria stained gram negative with bipolar ends. Beta – hemolysis was observed around colonies when grown on blood agar plates.

Earlier to this study, there were few published results of vaccine viability under long term storage at various temperature conditions. According to one study (7), a fowl pox vaccine stored for 21 years at 40°F was demonstrated to protect all birds subsequently challenged with FPV, an ILT vaccine stored for 25 years at 40°F showed protection of 40% of inoculated birds, and a pigeon pox vaccine stored for 20 years at 40°F protected all inoculated birds (7). Another study in particular performed assays on a vaccine for a 2-year period only to oversee stability at -20°C (11). Accelerated stability testing of vaccines is routinely done by storing vaccines at 37°C (15). In another report, NDV freeze-dried vaccine strains kept at 4°C over the course of 2, 4, and 6 months had little difference in titer (2). Strains kept at 37°C for a total observation period of 21 days had a notable change in titer (2). Yet in another report, freeze-dried vaccines were exposed to temperatures of 20°C, 4°C, and -20°C and then titered (13). The testing of a freeze-dried vaccine (Pneumomune) at 5.6°C, 21°C, and 37°C exhibited a drop of 1 to 2 logs in titer at 21°C and 37°C within a 24-hour period (17). Stability of a specific rotavirus vaccine was reported to be maintained at 37°C and 40°C for 18 months in addition to enduring alternating cycles of -20°C and 42°C (10). Loss of infectivity rates were tested for NDV, FPV, IBV, and ILTV vaccines in another study to measure stability (8). Controls at 3°C versus 37°C storage

found NDV was inactivated after 10 months and IBV at 6 months (8). It was observed that ILTV had a loss of titer by 2 logs after 3 years at 37°C and FPV had a loss of titer by 2 logs after 2 years (8). Further testing of an NDV variant was shown to be stable at 18-22°C for 3 months before losing titer by 1 log after 3 more months (12).

It appears that all of these reported studies were for a relatively shorter period of storage compared to the current study. Our study serves to add to the growing body of literature of vaccine virus viability concerning vaccines that have been in storage for several decades. No such studies were conducted earlier to evaluate the viability of vaccines stored for more than 30 years. Our findings provide evidence for the viability of vaccines spanning 24 - 33 years in storage while withstanding a break in the cold storage chain.

CONCLUSION

The lyophilized vaccines stored for a period of 24 – 33 years past the expiration date were successfully passaged in SPF embryonated eggs and cell cultures to check for virus viability. The revived viruses were titered to quantify the amount of recoverable virus. We have shown that all the stored lyophilized vaccines revived are capable of retaining viability even two to three decades past the expiration date and storage under suboptimal conditions for various periods of time. These findings are helpful in informing conditions for proper storage and shipping of vaccines in differing climates subject to drastic changes in temperatures.

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Table 1. Storage history of the vaccines used in the study.

Vaccines	Date of expiration	Storage temperature					Total storage period since expiration (years)	Date of testing
		2-7°C (years) ⇨	Room temp (days) ⇨	2-7°C (years) ⇨	-20°C (years) ⇨	2-7°C (years) ⇨		
ND	Oct/1990	18	2	2	10	2	32	OCT/2022
ILT	Nov/1997	11	2	2	10	2	25	OCT/2022
FP	Oct/1990	18	2	2	10	2	32	OCT/2022
IBD	Aug/1989	19	2	2	10	2	33	OCT/2022
Teno-1133	Jul/1998	10	2	2	10	2	24	JAN/2022
IBV -Conn	Mar/1993	15	2	2	10	2	29	JAN/2022
IBV-(M48)	Aug/1996	12	2	2	10	2	26	JAN/2022
PM	Apr/1990	18	2	2	10	2	32	JAN/2022

⇨ = Changes in the storage period of the vaccines at different temperatures

Table 2. Results of virus passage in embryonated eggs and cell cultures.

Vaccines tested	Total period of storage at various temperatures (years)	Egg inoculation results (virus induced lesions)	Cell passage results (virus induced CPE)	Virus titer at revival (log10EID50/ mL)	Conclusion
ND	32	Yes	NA	6.1	Viable
ILT	25	Yes	NA	3.5	Viable
FP	32	Yes	NA	6.2	Viable
IBD	33	NA	Yes	NA	Viable
Teno-1133	24	Yes	NA	7.3	Viable
IBV- Conn	29	Yes	NA	5.2	Viable
IBV- M48	26	Yes	NA	6.2	Viable

NA= Not applicable

VACCINATION WITH *SALMONELLA* SIDEROPHORE RECEPTOR AND PORIN (SRP®) PROTEIN VACCINES INDUCE PROTECTION AGAINST HETEROLOGOUS CHALLENGE

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ABSTRACT

Multiple proteins are present in the outer membranes of bacteria, but not all are good immunogens that induce adequate host immune response and resulting protection. Siderophore receptor and porin (SRP®) proteins are critical for the survival of pathogenic bacteria and are good targets for the host immune response. These siderophore receptor proteins are conserved among bacterial serotypes as evidenced by the cross-reaction of antibodies induced by *Salmonella* Enteritidis (SE) SRP vaccination with siderophore receptors of other *Salmonella* serotypes. Studies were conducted to determine whether vaccination with SE SRP vaccines can protect against heterologous *Salmonella* challenge. Studies in the USA and Brazil showed that vaccination with SE SRP was able to prevent tissue colonization after *Salmonella* Typhimurium (ST) and *Salmonella* Infantis (SI) challenge. Mortality after heterologous challenge with *Salmonella* Gallinarum (SG) was significantly reduced by SE SRP vaccination. These studies show that the use of SRP SE vaccines provides some cross-protection against heterologous challenges.

INTRODUCTION

Vaccination, as part of a sustained comprehensive risk reduction practice, is routinely used to prevent *Salmonella* infection in long-lived poultry. Together with effective biosecurity to keep the flock and environment *Salmonella*-free, good farm management, thorough cleaning and disinfection, and vaccination helps reduce the risk of *Salmonella* transmission to progeny or the contamination of eggs for human consumption (1, 2). Live *Salmonella* vaccines are administered to poultry at a young age to elicit immunity and provide early protection. Live vaccines induce both humoral and cell-mediated immune responses that provide protection against homologous and heterologous *Salmonella* serotypes (3, 4). Inactivated *Salmonella* bacterins are used to stimulate strong humoral immune response that provides systemic protection during the lay period. Whole cell bacterins, however, induce serotype-specific response to its own LPS or O-antigens and do not protect against heterologous challenge (3, 5). Other antigens, e.g., pili, flagella, outer membrane proteins, have been tried as alternatives to live and inactivated *Salmonella* vaccines (6). However, most have not provided levels of protection adequate for regulatory approval and commercial use.

Siderophore receptor and porin (SRP®) proteins are found traversing through the outer membrane of cells (7, 8). As the name signifies, these proteins serve a critical function in the entry of iron-bearing siderophore compounds into the cells. Iron is a critical nutrient in the survival of cells and therefore disruption of siderophore receptor function will result in starvation of iron and death of the cells (9). Furthermore, the attachment of circulating antibodies to siderophore receptors of the challenge bacteria result in opsonization and increased phagocytosis (10).

SRP protein antigens from *Salmonella* Enteritidis (SRP SE) were used to develop a new type of inactivated *Salmonella* vaccine. Vaccination with SRP SE have been shown to protect against liver, spleen and reproductive tissue colonization by homologous SE challenge (11, 12). These SRPs are conserved within *Salmonella* serotypes and other Gram-Negative bacterial species (*E. coli, Klebsiella*) (7). SDS-PAGE analysis of various avian *Salmonella* serotypes show that they all share 3-4 siderophore receptors proteins. Serum antibodies from birds vaccinated with SRP SE vaccines were found to cross-react with SRPs of other *Salmonella* serotypes in a Western Blot assay. This cross-reaction was further observed in Rapid Plate Agglutination assays. Sera from SRP SE-vaccinated birds cross-reacted with other *Salmonella* serotypes. (unpublished data)

Based on these *in vitro* studies, a series of vaccination-challenge studies were conducted to determine whether antibodies elicited by SRP SE vaccination would also protect against challenge with heterologous *Salmonella* serotypes.

MATERIALS AND METHODS

The first study was conducted in 15-week-old SPF hens divided into Vaccinated (n=26) and Control groups (n=27). SRP SE vaccine was administered subcutaneously to birds in the Vaccinated group at the recommended 0.25 mL/dose at 15- and 24-weeks of age. The birds in the Control group were vaccinated with placebo. The birds were commingled and challenged with nalidixic-acid-resistant *Salmonella* Typhimurium (ST) administering 1.0 mL orally and 1.0 mL intraperitoneally (1×10^6 CFU/mL) at 27 weeks of age. Liver, spleen, ovary, oviduct and cecal junction were sampled for the presence of the challenge ST at 29 weeks of age. The frequency of isolation of the challenge ST were noted in each group and Preventive Fraction analysis was used to determine the effectiveness of the vaccine.

A second study was conducted in a research facility in Brazil using 430 commercial brown egg layers (Lohmann Brown) to determine cross-protection provided by different *Salmonella* vaccination programs. The chicks were kept free of SE, ST and *Salmonella* Gallinarum (SG) and divided into 12 treatment groups (n=30 or 50/group) based on the different vaccines and programs used and *Salmonella* challenge, i.e., Non-Vaccinated/SE-Challenged (NV/SEC), Non-Vaccinated/ST-Challenged (NV/STC), Non-Vaccinated/SG-Challenged (NV/SGC), SRP SE-Vaccinated/SE-Challenged (SRP/SEC), SRP SE-Vaccinated/ST-Challenged (SRP/STC), Subcutaneously SRP SE-Vaccinated/SGC-Challenged (SSRP/SGC), Intramuscular SRP SE-Vaccinated/SG-Challenged (MSRP/SGC), SE+SG-Vaccinated/SG-Challenged (SEG/SGC), Live SG 9R + SE+SG Vaccinated/SG-Challenged (9RSEG/SGC), Live SG 9R + SRP SE Vaccinated/SG-Challenged (9RSRP/SGC), Live SG 9R-Vaccinated/SG-Challenged (9R/SGC), and, Non-Vaccinated/Non-Challenged (NV/NC). SRP SE and SE+SG bacterins were administered at 9- and 14-weeks of age subcutaneously or intramuscularly. Live SG 9R vaccines were administered at 7- and 10-weeks of age. Two of the SG 9R groups were also vaccinated with either SRP SE or SE+SG bacterin at 14 weeks. All birds were challenged at 18 weeks of age with either SE (2.2×10^8 CFU/mL), ST (3.5×10^8 CFU/mL) or SG (1.5×10^8 CFU/mL) orally. Level of systemic bacterial infection was determined by humanely sacrificing birds and sampling their liver, spleen and ceca at 4-, 7-, 11-, 14-, 21- and 28-days post-infection. For the SG challenged groups, mortality was recorded throughout the 2-week observation period. Level of SE or ST colonization in the vaccinated groups were compared with their corresponding non-vaccinated groups. For the SG-challenged groups, livability or prevention of mortality from challenge was compared.

In the third study, six-week-old SPF pullets were divided into four vaccination and two non-vaccinated controls groups (n=32/group), i.e., commercial whole cell SE bacterin, whole cell autogenous SI bacterin, commercial SE SRP vaccine, autogenous SI SRP, Unvaccinated Challenged group, and, Unvaccinated Unchallenged Control group. At 11-weeks of age, the birds were challenged with either the corresponding homologous or heterologous SE or SI at a dose of 10^7 CFU/0.2 mL intravenously. Ten birds/group were humanely sacrificed at 7-, 14- and 21-days post-infection and levels of the challenge *Salmonella* infection were determined from the spleen and feces. Protection from homologous and heterologous challenge were compared between the different vaccinated groups.

RESULTS

The study in 15-week-old SPF hens demonstrated that vaccinating twice with SRP SE vaccines even with 9-weeks gap in-between administrations was able to protect against heterologous ST challenge. Colonization of the liver/spleen and oviduct of birds from the Vaccinated group were significantly ($P \leq 0.05$) less compared to birds from the Control group, i.e., 0% vs. 22.22% in the liver/spleen and 0% vs. 21.74% in oviducts, 14 days post-challenge (Figure 1).

The Brazilian study in commercial brown egg layers supported the cross-protective efficacy of SRP SE vaccination when challenged with heterologous ST. Level of ST infection in the cecal samples from the Vaccinated group were reduced on Days 7 (1.1 Log_{10} CFU/g) and 11 (1.7 Log_{10} CFU/g) post-infection compared to the Control birds (Figure 2). No significant differences were noted in the other tissues and other time points. Birds vaccinated with SRP SE protected against SG challenge reducing mortality in vaccinated birds significantly ($P \leq 0.05$) compared to non-vaccinated and SG-challenged birds..

The third study confirmed that whole cell bacterins protected against homologous SE or SI challenge. Level of colonization in the spleen and feces were reduced significantly on Days 7, 14 and 21 post-challege ranging from 1.8-4.3 Log_{10} CFU/g in the SE-challenged groups and from 1.1-3.6 Log_{10} CFU/g in the SI-challenged groups. However, the whole cell SE bacterin did not protect against heterologous SI challenge at any time. Autogenous whole cell SI

bacterin provided minimal protection against heterologous SE challenge reducing colonization in spleen and feces from 0.5-0.9 Log_{10} CFU/g. Single administration of SRP SE or SRP SI vaccines protected against both homologous and heterologous challenge. No challenge SE or SI bacteria were re-isolated from spleen and feces of SRP-vaccinated birds at any time point after challenge (Table 1).

DISCUSSION

The use of live and inactivated *Salmonella* vaccines is critical in the success of *Salmonella* control and food safety programs in poultry. Due to the numerous serotypes that challenge especially cage-free and floor-raised flocks, control of both homologous and heterologous *Salmonella* challenge is essential for vaccination programs to succeed. Siderophore receptor and porin (SRP) proteins were selected as immunogens because of their conserved presence across *Salmonella* serotypes and the critical function in bacterial viability.

The ability of antibodies induced by SRP SE vaccines to cross-react with SRPs or antigens of other *Salmonella* serotypes were demonstrated using in vitro Western Blot and Plate Agglutination assays. A series of vaccination-challenge studies in both SPF and commercial brown egg layers demonstrated that vaccination with SRP SE vaccines protected against ST, SG and SI challenge in controlled environments. SRP SE vaccination reduced the level of infection or colonization of selected tissues after ST and SI challenge compared to non-vaccinated or whole-cell bacterins. SRP SE vaccination reduced mortality after SG challenge compared to non-vaccinated controls.

These series of *in vitro* and *in vivo* studies demonstrated that analogous to live *Salmonella* vaccines, siderophore receptor and porin (SRP) protein vaccines protect against both homologous and heterologous challenge. Whereas, protection provided by whole cell bacterins were limited to homologous challenge. These studies provide evidence that SRP vaccines can be an effective component of successful *Salmonella* vaccination programs in poultry.

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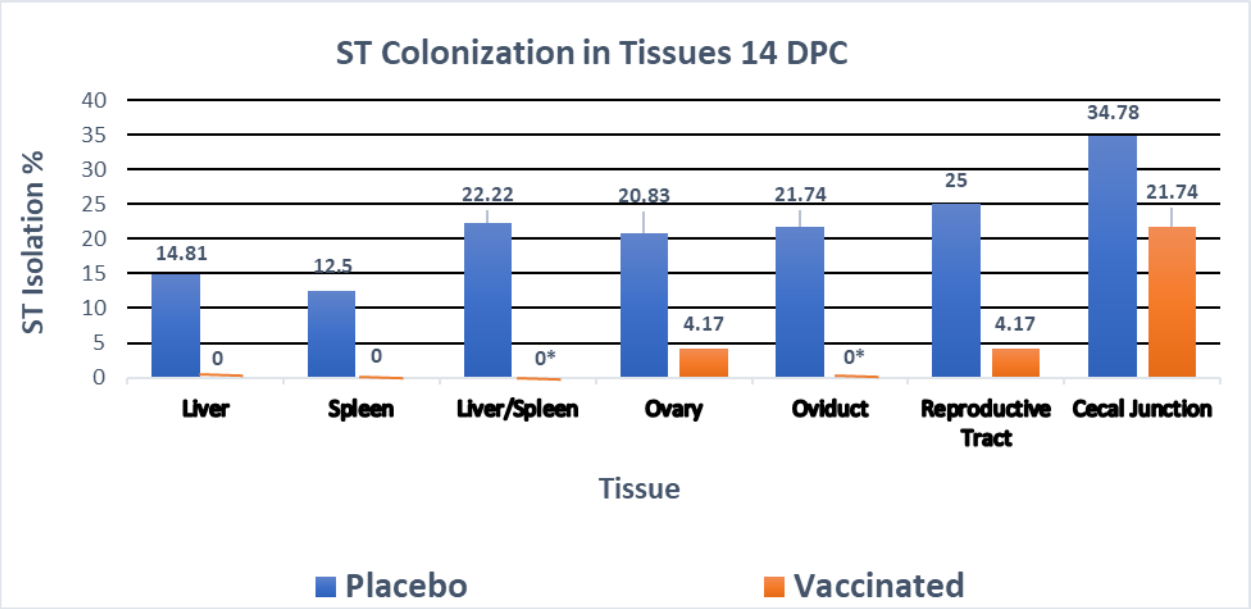
Figure 1. Level of colonization of *Salmonella* Typhimurium (ST) 14 days after challenge in birds vaccinated with Placebo and *Salmonella* Enteritidis siderophore receptor (SE SRP) protein vaccine.



* *P≤0.05*

Figure 2. Level of *Salmonella* Typhimurium in cecal contents of birds vaccinated with *Salmonella* Enteritidis siderophore receptor protein (SE SRP) vaccine and non-vaccinated control.

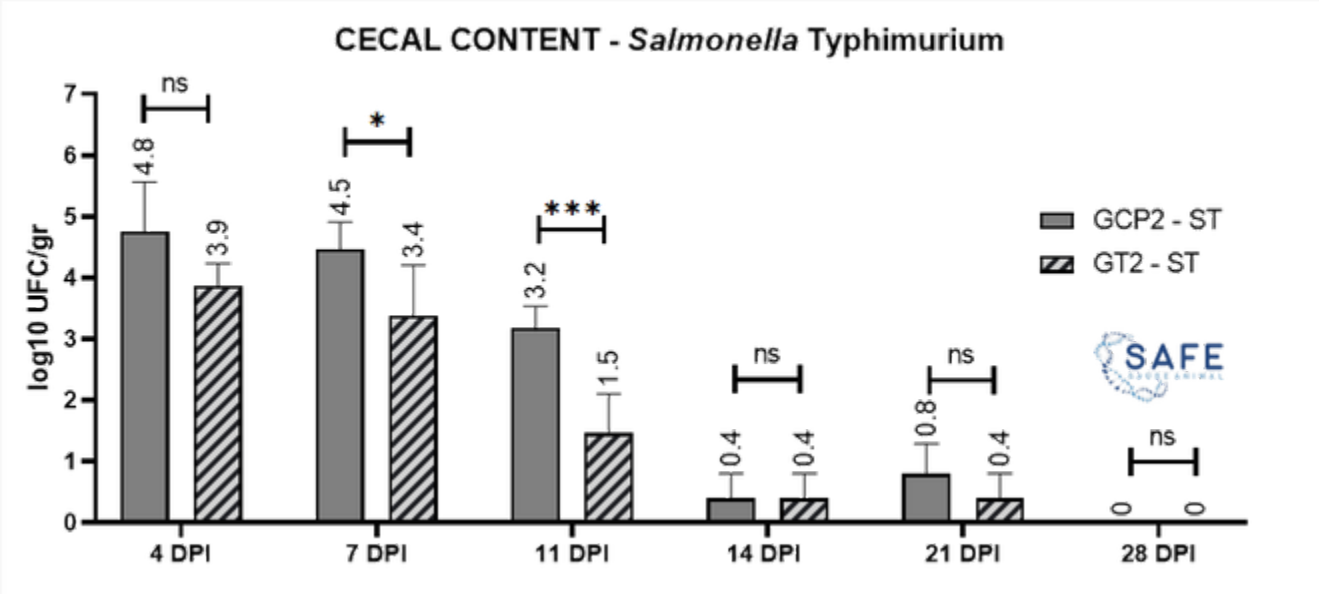


Table 1. Recovery of *Salmonella* Infantis (SI) challenge in spleen and feces at 7-, 14- and 21-days post-challenge in unvaccinated birds and birds vaccinated with whole cell *Salmonella* Enteritidis bacterin, whole cell *Salmonella* Infantis bacterin, SRP SE and SRP SI vaccines.

S INFANTIS (SI) CHALLENGED Treatment Group	7 dpc				14 dpc				21 dpc	
	Spleen		Feces		Spleen		Feces		Feces	
	Pos/ Total	Avg Log CFU/g	Pos/ Total	Avg Log CFU/g	Pos/ Total	Avg Log CFU/g	Pos/ Total	Avg Log CFU/g	Pos/ Total	Avg Log CFU/g
Unvaccinated Unchallenged	0/0	0	0/0	0	0/0	0	0/0	0	0/0	0
Unvaccinated Challenged	9/9	6.2[a]	10/10	6.3[a]	10/10	4.9[a]	10/10	4.8[a]	10/10	4.3[a]
(A) WCB SE Commercial	10/10	6.3[a]	10/10	6.2[a]	7/10	4.5[b]	9/10	4.4[b]	10/10	4.1[b]
(B) WCB SI Autogenous	7/10	2.6[b]	7/10	2.7[b]	8/10	3.0[c]	8/10	3.0[c]	10/10	3.2[c]
(C) SRP SE Commercial	-	-	-	-	0/5	0[d]	0/5	0[d]	-	-
(D) SRP SI Autogenous	0/10	0[c]	0/10	0[c]	0/10	0[d]	0/10	0[d]	0/10	0[d]

[abcd] Statistically significant (P<0.05)

BLOOD SAMPLE-BASED PCR ASSAY FOR *HISTOMONAS MELEAGRIDIS*: WHAT HAVE WE LEARNED SO FAR?

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SUMMARY

Histomoniasis is a fatal disease of turkeys caused by the protozoan parasite, *Histomonas meleagridis*. The diagnosis of histomoniasis is based on the gross and histopathology lesions. The clinical signs and mortalities are usually seen at least 1-2 weeks after the onset of infection. With the objective to develop an early diagnostic tool for detection and confirmation of histomoniasis, two experimental studies were conducted. In experimental study 1, two pens (20 birds/pen) were challenged at 14 DOA by intra-cloacal route. Four days post-challenge, blood samples were collected. DNA was extracted from the blood samples and PCR was done with *H. meleagridis* primers for the 18S rRNA gene. Fifty-five percent of the samples had positive bands for *H. meleagridis* in the PCR gel and confirmed by sequencing. In experimental study 2, one group (20 birds/group) was challenged at 14 DOA by intra-cloacal route, while a second group served as unchallenged control. Two days post-challenge, blood samples were collected. Five percent of the samples were confirmed as positive for *H. meleagridis* based on PCR and sequencing. These results indicate that *H. meleagridis* can be detected and confirmed in the blood as early as two days after infection.

INTRODUCTION

Histomoniasis is a fatal disease of turkeys caused by the protozoan parasite, *Histomonas meleagridis*. The clinical signs of histomoniasis are usually seen around 6-12 days after the onset of infection (1). In experimental studies, early mortalities may be noticed after 6 days of infection, while peak mortality is noticed around 13-15 days (2). Therefore, in field outbreaks, the diagnosis of histomoniasis based on the gross and histopathology lesions could be made at least 1-2 weeks after the onset of infection. By the time of disease confirmation the infection might have spread to neighboring flocks. Thus, early diagnosis of *H. meleagridis* would benefit the poultry industry by allowing rapid implementation of strict biosecurity and control measures.

Evaluation of the serum samples in our previous experimental studies revealed that cholesterol and serum alkaline phosphatase (ALKP) were significantly decreased in *H. meleagridis* challenged turkeys compared to the unchallenged turkeys (3, 4). A decrease in cholesterol and ALKP can be considered as a surrogate marker, but does not confirm the presence of *H. meleagridis* infection in turkeys. In this report, two experimental studies were conducted to investigate blood samples from turkeys challenged with *H. meleagridis*.

MATERIALS AND METHODS

Experimental Study 1. Forty day-of-hatch female poults were received from a local hatchery. The turkeys were housed in two floor pens (20 birds/pen). At 14 DOA, all birds were challenged with *H. meleagridis* (HMB) at the dose of $1x10^4$ histomonads/0.5 mL dose by intra-cloacal administration. Four days post-challenge, all birds were bled and anticoagulated blood samples were collected. All birds were necropsied at 15 days post-challenge.

Experimental Study 2. Forty day-of-hatch female poults were divided into two groups and housed in two floor pens (20 birds/pen). At 14 DOA, one group was challenged with *H. meleagridis* (HMB) at the dose of $1x10^4$ histomonads/0.5 mL dose by intra-cloacal administration, while the other group served as negative control. Two days post-challenge, all birds were bled and anticoagulated blood samples were collected. All birds were necropsied at 14 days post-challenge.

Gross lesions. Any mortalities that occurred after the day-of-challenge were evaluated for gross lesions in ceca and liver. The ceca and liver were scored from 0-4 based on the severity of the lesions.

Molecular diagnostics. DNA was extracted from the anti-coagulated blood samples using DNeasy Blood and Tissue Kit (Qiagen). Thirty µl of proteinase K (Qiagen) was used to digest 100 µl of blood sample. PCR was performed against *H. meleagridis* 18S rRNA gene (5). The PCR products were purified and submitted for sequencing.

RESULTS

Experimental Study 1. Based on the mortalities and gross lesions in the ceca and liver, an overall infectivity rate of 67.5% (27/40) was documented in both pens at 15-days post-challenge. *H. meleagridis* DNA was detected and confirmed in 55% (22/40) of the blood samples from both pens, collected at 4 days post-challenge.

Experimental Study 2. An overall infectivity rate of 95% (18/19) was documented in the challenged group at 14 days post-challenge with *H. meleagridis*. *H. meleagridis* DNA was detected and confirmed in 5% (1/20) of the blood samples in the challenged group collected at 2-days post-challenge.

DISCUSSION

Successful therapeutic treatments for histomoniasis were only achieved in cases where the treatment was started at an early stage of the disease (6). Therapeutic treatment initiated after 1-2 weeks of increased mortalities offered only limited protection (7). Thus, an early diagnostic tool is needed to detect *H. meleagridis* infection in flocks. The systemic spread of *H. meleagridis* has been reported before (8, 9) and this detection method relies on the presence of *H. meleagridis* DNA in the blood samples. The 18S rRNA PCR primers are highly sensitive and not specific for *H. meleagridis* (5). In these studies, the confirmation of *H. meleagridis* was made only after sequencing and not just based on the visualization of the amplicons in the PCR gel. Based on the results of two experimental studies, *H. meleagridis* DNA can be detected in blood samples as early as two days post-challenge. As the disease progresses, an increased number of positive samples were detected at four days post-challenge. Application of this detection method in histomoniasis field outbreaks may help in earlier detection of the disease.

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HISTOMONIASIS IN ORGANIC TURKEYS AND ITS ECONOMIC IMPACT

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SUMMARY

Increased incidence of histomoniasis in poultry, especially turkeys, has been reported in recent years due to the lack of prophylactic and therapeutic measures to control the disease. In British Columbia, Canada, for instance, histomoniasis outbreaks in turkeys increased in 2020 compared to 2019 and the years before. We conducted several field studies to investigate the epidemiology, pathogenesis and economic impact. This case study details a histomoniasis outbreak in 35-day-old organic turkey hens (n=3500) in Canada. The assessed farm had a history of histomoniasis and gross pathology investigation and histopathological evaluation confirmed its presence in the current case. Genotyping of 18S rRNA revealed that *H. meleagridis* genotype-1 was involved in this outbreak. The economic losses were calculated for this flock (n=3,500). In addition, economic losses were also estimated for a hypothetical typical mid-size flock (n=10,000). The histopathology, gene targeted sequencing and economic losses were studied in this histomoniasis outbreak. To the best of our knowledge, this is the first case report of histomoniasis in organic turkeys with estimated economic impact.

INTRODUCTION

Histomonas meleagridis, a flagellated protozoal parasite, causes histomoniasis in gallinaceous birds. Among the gallinaceous birds, turkeys are highly susceptible, and the fatality rate can reach up to 80-100% (1). Histomoniasis causes dullness, depression, cachexia, ruffled feathers, listlessness, droopy wings, sulfur-yellow feces, and yellow stained feathers around the vent region. Recently, increased incidences of histomoniasis have been reported due to the lack of commercial vaccines and prophylactic/therapeutic measures (1). In British Columbia, Canada, histomoniasis incidences increased to 200% in 2020 compared to 2019 and resulted in more than CAD$985,000 losses (2). It is estimated that histomoniasis associated annual losses exceeds USD$2 million globally (3). In an effort to support the turkey industry, field studies were conducted in histomoniasis outbreaks in USA (4) and Canada (5) to understand the epidemiology, pathogenesis and potential economic impact. This case report details the histomoniasis outbreak in organic turkeys.

CASE REPORT

In Summer 2021, a histomoniasis outbreak was reported in a two-stage organic turkey hen farm (n=3,500) located in Canada. The outbreak was reported at 35-days-of-age and samples were collected at 44 days-of-age. Thirty percent mortality was reported and 50% of the remaining flock had clinical signs of histomoniasis. Cecal samples were collected and stored in 10% neutral buffered formalin and liver samples were collected and stored on ice.

MATERIALS AND METHODS

Cecal samples in 10% neutral buffered formalin were paraffin embedded. Sections of paraffin embedded cecal tissues were stained with hematoxylin and eosin following standard histologic procedures. The stained slides were evaluated following standard histologic procedures. DNA was extracted from the liver samples by using DNeasy® Blood & Tissue Kit (Qiagen) by following manufacturer's instructions. A PCR was performed targeting the 18S rRNA gene of *H. meleagridis* as described previously (6) and sequenced. The economic losses were calculated for this flock (n=3,500) based on the information derived from turkey producers in Canada. In addition, economic losses were also estimated for a hypothetical typical mid-size flock (n=10,000).

RESULTS

Gross Pathology. Necropsy findings include necrotizing typhlitis, markedly enlarged ceca filled with caseous cores and multifocal necrotic hepatitis. Based on the gross pathology lesions, a presumptive diagnosis of histomoniasis was made.

Histopathology. In necropsied turkeys, all cecal sections had moderate to marked, pyogranulomatous inflammation and necrosis extended through all the layers of the intestine. The ceca lumen contained abundant fibrinocellular debris infiltrated with moderate to abundant hemorrhage, degenerated heterophils, mucosal epithelium, abundant pleomorphic bacteria and *Histomonas* trophozoites. Ceca mucosa and submucosa were expanded by inflammation, abundant *Histomonas* trophozoites, edema, hemorrhage, and degenerate cellular debris. In the most severely affected sections, trophozoites infiltrated all layers of the intestine and adjacent mesentery. The intestinal serosa, mesentery and caudal air sacs were to abundantly thickened by mesothelial hypertrophy, air sac epithelium hyperplasia and hypertrophy, granulation tissue, heterophilic and histiocytic inflammation, and variably organized lymphoid tissue.

Economic analysis. Considering the expenses for poult, organic feed, fixed and variable costs including labor, insurance etc. for a flock size of 3,500 turkeys, an approximate profit of CAD$ 36,054 was expected. With 30% mortality rate, the turkey producer would lose CAD$6,846 out of pocket with a total loss of CAD$42,900 (36,054+6846). With an 80% mortality rate, the turkey producer would lose CAD$77,892 out of pocket with a total loss of CAD$113,946 (36,054+77,892).

For a typical mid-size flock (n=10,000), an approximate profit of CAD$82,687 was expected. With 30% mortality, the turkey producer would lose CAD$7,565 out of pocket with a total loss of CAD$90,252 (82,687+7, 565). With 80% mortality rate, the turkey producer would lose CAD$220,577 out of pocket with a total loss of CAD$303,264 (82,687 +220,577).

DISCUSSION

Field studies provide valuable information in understanding the circulating isolates and possible critical factors and concurrent infections involved in histomoniasis outbreaks (4,5). Based on the gross lesions, a preliminary diagnosis of histomoniasis was made in this field study. This was confirmed by the presence of *Histomonas* trophozoites on histopathology. Molecular techniques confirmed the genotype, *Histomonas meleagridis* genotype-1, based on the analysis of 18S rRNA sequences. The assessment of field studies can therefore play a vital role in understanding the pathogenesis of the disease and providing information on circulating strains. In addition, a detailed economic analysis was conducted to understand the potential loss associated with histomoniasis outbreaks. With an 80% mortality rate, a small-size flock with 3,500 organic turkeys, such as current field case, would incur a loss of CAD$113,946, while a hypothetical mid-size flock with 10,000 organic turkeys would incur a loss of CAD$303,264. To the best of our knowledge, this is the first clinical case report of histomoniasis in organic turkeys with estimated economic analysis report considering all possible attributes.

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PROPHYLACTIC TREATMENT OF PAROMOMYCIN (LIQUID FORM) AGAINST *HISTOMONAS MELEAGRIDIS* REDUCES MORTALITY AND GROSS LESIONS

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SUMMARY

Lack of prophylactic and therapeutic measures to control histomoniasis has resulted in increased field outbreaks globally. The objective of our study was to evaluate the prophylactic efficacy of liquid form of paromomycin (Huvepharma NV, Belgium) against histomoniasis in turkeys. Paromomycin is an aminoglycoside antibiotic and had been found efficacious against various protozoa. Four groups were included in this study with 20 poults/group. Groups 1 and 2 served as the negative and positive controls of this study, respectively. Group 3 received paromomycin in drinking water from 12-18 days-of-age (DOA) at the dose of 1.25 mL/kg body weight (7-days treatment). Group 4 received paromomycin in drinking water from 12-27 DOA at the dose of 1.25 mL/kg body weight (15-days treatment). At 14 DOA, groups 2-4 were challenged with $1x10^4$ *H. meleagridis*/0.5 mL dose by intra-cloacal route. At 28 DOA, all the birds were necropsied and evaluated for cecal and liver lesions. Group 2 (challenge control) had 95% mortalities and gross lesions of histomoniasis. Group 3 (paromomycin-7 days treatment) had 5% mortality and 35% incidence of gross lesions due to histomoniasis. Group 4 (paromomycin-15 days treatment) had 10% mortality and 15% incidence of gross lesions due to histomoniasis. This study demonstrates that the prophylactic treatment of paromomycin offers protection against histomoniasis by reducing the mortality rate and the incidences of gross lesions in ceca and liver.

INTRODUCTION

Histomonas meleagridis, a protozoan parasite, causes histomoniasis (blackhead disease, histomonosis) in gallinaceous birds. Histomoniasis is a fatal disease of turkeys causing up to 80-100% mortalities (1). Ban and withdrawal of effective prophylactic and therapeutic products resulted in increased outbreaks of histomoniasis globally (2,3,4,5). There are no commercial vaccines available to prevent histomoniasis infection in turkeys. Thus, there is a need to identify an alternative solution for histomoniasis. Paromomycin is an aminoglycoside antibiotic that binds to 3' end of single-stranded rRNA of various protozoa such as *Trichomonas*, *Giardia*, *Entameoba* and *Histomonas* (6, 7, 8, 9). In this study, we evaluated the prophylactic treatment of paromomycin against *H. meleagridis* challenge.

MATERIALS AND METHODS

Experimental study. Eighty day-of-hatch poults were obtained from a local commercial hatchery and housed in four floor pens (20 birds/group). Poults were identified by individual numbers on their neck-tags. Four groups were enrolled in this study. The groups were 1) negative control (birds were not challenged with *H. meleagridis*), 2) challenge control (birds were challenged with *H. meleagridis*), 3) paromomycin-7 days treatment at the dose of 1.25 mL/kg BW (birds were challenged with *H. meleagridis* after 2 days of treatment) and 4) paromomycin-15 days treatment at the dose of 1.25 mL/kg BW (birds were challenged with *H. meleagridis* after 2 days of treatment). At 14 DOA, groups 2-4 were challenged with wild-type *H. meleagridis* isolate (HMB) at the dose of $1x10^4$ histomonads/0.5 mL dose by intra-cloacal route. Paromomycin has the active ingredient of paromomycin sulfate. One mL of paromomycin liquid has 200 mg paromomycin sulfate which is equivalent to a paromomycin base of 140 mg or 140,000 IU of paromomycin activity. At 28 DOA, all the birds were necropsied and evaluated for cecal and liver lesions.

Gross lesions. Gross lesions in the ceca and liver were assigned based on the severity of the lesions. Cecal lesions were assigned from 0 (no visible lesions), 1 (indistinct hemorrhage or few thickened foci), 2 (distinctly thickened ceca with hemorrhage or ulcers, absence of cecal core), 3 (solid cecal core with distinctly thickened ceca along with hemorrhage or ulcers) and 4 (necrotic and fibrinous ceca with fibrinous cecal core ± severe hemorrhage). Liver lesions

were assigned from 0 (no visible lesions), 1 (indistinct necrotic foci in the liver n<5), 2 (distinct necrotic foci in <25% of the liver surface), 3 (necrotic foci noticed in 25-75% of the liver surface) and 4 (necrotic foci noticed in more than 75% of the liver surface).

Statistical analysis. ANOVA was used to analyze the significant differences between the initial body weight and the average body weight. A Kruskal-Wallis test was performed on the cecal and liver scores. The incidences associated with cecal and liver gross lesions and mortalities were evaluated by Fisher's Exact Test. Statistical differences were considered at a significance level of $p \leq 0.05$.

RESULTS

Group 2 (challenge control) had 95% mortalities, while groups 3 and 4 (paromomycin treatments) had 5 and 10% mortalities, respectively. Group 2 had mean liver lesion score (MLLS) of 3.00±1.25, while groups 3 and 4 had lower MLLS of 0.65±1.39 and 0.40±1.05, respectively. Group 2 had mean cecal lesion score (MCLS) of 3.52±1.12, while group 3 had lower MCLS of 0.75±1.33. No gross lesions were noticed in the ceca of birds in group 4.

DISCUSSION

Following biosecurity measures and several control measures alone cannot prevent histomoniasis outbreaks (5,10). Since the therapeutic effects of paromomycin is limited, there is a desperate need for prophylactic use of paromomycin (2, 5). The prophylactic treatment of paromomycin administration in the drinking water resulted in decreased mortality rates and increased the survivability. In addition, the MCLS and MLLS were significantly decreased in the paromomycin treatment groups compared to the challenge controls. Oral administration of paromomycin is poorly absorbed in the digestive tract and more than 99% of the drug is excreted in feces (11, 12, 13). Since paromomycin is retained in the feces, it is an added advantage so that the ceca is likely to be protected at the entry level of the pathogen. Since, paromomycin can be administered in drinking water, mass administration can be easily achieved. Thus, administration of paromomycin in drinking water is efficacious against histomoniasis in turkeys.

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THE STEALTH PROTOZOAN PARASITE OF GALLINACEOUS BIRDS

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SUMMARY

Histomoniasis or blackhead is a pathogenic disease of gallinaceous birds. Turkeys are extremely susceptible to the disease, resulting in high mortality at times. Histomoniasis appears to be less pathogenic to chickens. This disease is of considerable economic importance to poultry producers due to reduced productivity of the affected flocks. Currently, there are no approved prophylactic and therapeutic products. In addition, there may be an incomplete understanding of these agents.

INTRODUCTION

Histomoniasis (Blackhead) has become one of the important protozoan diseases in poultry. The causative agent is a flagellated protozoon Histomonas meleagridis. Gallinaceous birds (turkeys, chickens, quail, and other fowls) are susceptible; however, non-gallinaceous birds such as ducks and ostriches may have some susceptibility. Turkeys are the most susceptible species to H. meleagridis. A review by Lund (1), provided an overview of the disease "blackhead" which highlighted the major contributors that had identified and unraveled some of the mysteries of the disease. McDougald (2), acknowledged the additional improvement in the understanding of the disease. However, he noted that after 28 years there was still a lack of research addressing control of the disease. Clark and Kimminau (3) agreed that not enough is being done to understand the pathogenesis and epidemiology of the disease and to obtain new products for control.

MATERIALS AND METHODS

Several evaluations were conducted to gain some insight into the Histomonad organisms. Litter samples from two different pullet farms with prior experiences of Blackhead were submitted. One farm had been out of birds for several months whereas the second farm had maintained a bird population. The samples were kept separately from collection through storage. In the first exercise, young chickens and turkeys were kept in wire-floor pens. A portion of the floor was covered with cardboard to provide a solid flooring. On the covered flooring, 200grams of litter from each source was placed on the covered area. An aliquot of 60grams of litter was blended with 200grams of unmedicated broiler ration (20-22% protein) of either litter samples. Birds were provided with food and water ad libitum for 14 days. There were four pens with four birds per pen: two pens for each sample, one of chickens and one of turkeys. The unused portions of the original litter samples were maintained in the original shipping containers and kept in a cool room for six months.

In another demonstration, naïve birds (chickens or turkeys) were given an intra-cloacal inoculation with 0.5 mL of the mixture of cecal scrapings in 10 mL of buffered saline. The birds were provided unmedicated feed ad libitum throughout the trial. Birds were kept in wire floor cages and kept there for 14 days.

After storing for six months, the original litter samples were prepared as described above but the two samples were combined to produce one source of litter material. This combined sample was provided to two-week-old naïve birds (chickens and turkey) were placed on the confirmed contaminated material and provided food and water ad libitum. After five days of exposure, these birds were placed in new cages with the flooring covered by new pine shavings. Birds were provided with feed and water ad libitum and kept in those cages for 10 days. After the 10th day, those birds were humanely euthanized, and ceca assessed for lesions. Another set of naïve poults were place into those pens and were provided with fresh food and water ad libitum and kept for 14 days.

In another exercise, three-week-old poults were placed in wire flooring pens with four birds per pen. There were four pens with four birds per pen. In one pen, two birds were inoculated rectally with a 1.0mL of the cecal scraping prepared in 10mL of buffered saline. In the second group, only one bird was inoculated. These inoculated birds were allowed to co-mingle with the uninoculated pen-mates. The other two groups were kept as uninoculated.

The cadavers (mortality or euthanized) were necropsied, and wet mount smears were prepared from the duodenum, jejunum, ileum, and ceca. Smears were prepared by using a drop buffered saline on a slide, the scraping added and examined under 100x and 400x magnification.

A 3-gram sample from each pullet litters was placed in a clean 50mL test tubes and a 15mL saturated sugar solution was added to each tube and allow to sit undisturbed for two hours. A small sample was removed from the meniscus and placed on a cleaned microscope slide for the detection of worm eggs.

RESULTS

The mixture of feed and litter feed produced clinical signs in the populations by the 7th day. The signs became more pronounced by the 10th day. Sulfur colored dropping were also noted in all groups. Birds were euthanized and necropsied to assess the lesions. Two poults died from the direct exposure to the litter. Cecal lesions were seen in all poults, but liver lesions were only seen in the dead birds. For the chickens, moderate cecal lesions were noted in 25% of the birds. The lesions of the chickens were less severe compared to those of the poults.

For the intrarectal inoculations, all five poults had moderate to severe cecal lesions; three died from severe cecal and liver lesions. In the chickens given the rectal inoculation, four of the five chickens exhibited cecal lesions. For the third demonstration in which the litter samples were kept for six months and naïve birds became exposed, three of five chicks had mild to moderate cecal lesions. However, 100% of the poults had clinical signs with a 20% mortality. For the exercise with the co-mingling of the rectally inoculated poults with uninoculated poults; the group with 50% inoculated, all inoculated birds died due to severe lesions by the 10th day. At termination the other two birds had clinical lesions of histomoniasis. The group that had a 25% of the birds inoculated, 60% of exhibited lesions of histomoniasis but only the inoculated died from the disease. For the uninoculated groups, the group closest to the 50% inoculated those birds had mild cecal lesions. But the group furthest away had 25% of the birds with mild cecal lesions. At termination, there were no mortalities in the uninoculated groups.

Wet mount smears confirmed all birds with typhlitis had varying stages of the Histomonad organisms. No Eimeria stages were noted during the evaluations. Flotations of the litter samples did not reveal worm eggs.

CONCLUSION

The litter samples were from pullet farms; however, when naïve chickens or turkeys were exposed to the litter samples, they became positive for Histomoniasis. Birds exposed to the agents intra-rectally or ingestion, demonstrated clinical signs of the disease. These exercises demonstrated that there may be more to the epidemiology of Histomoniasis than is currently accepted.

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HOW DID THE STUDENT GET TO THE OTHER SIDE: FACTORS INFLUENCING CAREER CHOICES IN POULTRY MEDICINE

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SUMMARY

Poultry medicine courses are rare in the standard veterinary curriculum of most veterinary schools. Some schools may offer poultry or avian courses as electives, but these topics are not universally taught. There are few poultry veterinarians, and I was interested in learning how students become involved in poultry medicine. Therefore, the objectives of this study were to investigate the points in time that poultry health professionals were exposed to poultry and what factors influence their interest in pursuing a career in poultry medicine. To achieve these objectives, a survey was distributed to poultry health professionals, which asked a series of questions about their demographic, geographic, and educational background, their current professional situation, and future goals. The full-length manuscript detailing the findings is in progress.

MATERIALS AND METHODS

Subjects. We surveyed members of the AAAP.

The Survey. The survey was created with Google Forms and consisted of a variety of types of questions. It was distributed during late 2020.

RESULTS

Categories of data about respondent's include background, education, career choice. The participants were given the opportunity to describe their satisfaction and obstacles in poultry medicine.

DISCUSSION

This study showed that there may be a variety of factors contributing to poultry medicine. By identifying these factors, we can contribute to increasing poultry veterinarians.

VACCINATION MONITORING AND DIVA TESTING FOR H5 AVIAN INFLUENZA

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SUMMARY

The Influenza A virus is at the origin of infections in both humans and animals and can result in more or less severe forms depending on the strain. To control outbreaks and avoid mass cullings, as well as significant economic losses for farmers, vaccination of flocks is increasingly necessary given the epidemiology of H5 Highly Pathogenic Avian Influenza (HPAI) worldwide.

Vaccination with recombinant vaccines has developed progressively. Therefore, Innovative Diagnostics has chosen to develop tools capable of differentiating antibodies developed after infection by a field strain from the antibodies elicited by vaccination of the animals.

INTRODUCTION

Influenza viruses belong to the family Orthomyxoviridae and infect a variety of human and animal hosts. There are four types of influenza viruses: A, B, C and D; which are defined by the nature of their internal nucleocapsid antigen. Type A is the most conserved genus and can be further divided into subtypes based on their Hemagglutinin (H) and Neuraminidase (N) antigens. Eighteen H antigens (H1 to H18) and eleven N antigens (N1 to N11) have been isolated. Most avian influenza viruses (H1 to 16 subtypes) are low pathogenic (LPAI), such as H9, and are generally involved in co-infections with other avian viruses, which can lead to significant losses in poultry flocks. Some subtypes containing H5 and H7 are associated with highly pathogenic forms of the disease, with high mortality rate.

A current H5 Highly Pathogenic Avian Influenza (HPAI) strain has been circulating around the world since 2004 and has been responsible for dramatic poultry losses, wild bird mortalities and human cases. All viruses belonging to this current H5 strain share a common known ancestor (Goose/Guangdong/1/96 strain).

Vaccination is an essential tool for poultry disease control (in addition to biosecurity). For many years, conventional vaccines have been used. Today, innovations in poultry vaccinology include immunocomplex vaccines and vector vaccines and their advantages include: biosecurity related to hatchery administration, efficacy, ability to elicit passive immunity, and long-lasting immunity. In the last 5 years, successive waves of Influenza in Europe have prompted health authorities to review their vaccination strategy against this virus, and now more specifically against the H5 subtype. It is now necessary to develop better AI vaccines to deal with this disease. Several studies have shown the efficacy of AI vector vaccines with H5, H7 and H9 genes inserted into an HVT vector.

Innovative Diagnostics has developed a new ELISA test, ID Screen® Influenza H5 Indirect which is based on the H5 protein. This kit is an excellent tool for monitoring vaccination with conventional and recombinant vaccines as well as for the implementation of DIVA strategies (vaccinated animals may be monitored using this new kit, and naturally infected animals can be detected using the ID Screen® Influenza A Nucleoprotein Indirect**).**

MATERIALS AND METHODS

FLUH5S kit specificity study

The exclusivity was studied using 2 different influenza serum panels:

One coming from GD Deventer: sera generated from immunization with 2 different AI strains (H5N2 and H5N3) were tested as well as H6, H7 and H9 subtypes.

Another panel from the Friedrich-Loeffler-Institute (FLI). Sera were tested against: H2N5, H2N9, H3N1, H5N2, H5N3, H6N2, H7N1, H7N7, H10N4 and H11N6 subtypes.

Secondly, to study the measured specificity with the FLUH5S ELISA test, 320 sera from SPF birds (origin: France) and 100 sera from disease-free broilers (origin: Hungary) were tested.

Monitoring of birds vaccinated with Vectormune AI (rHVT-AI(H5))

Chicks from broilers were vaccinated with Vectormune AI rHVT-H5 vaccine (from Ceva Santé Animal) at one day of age. The animals were challenged at 55 weeks of age by HPAI H5N8 strain. Antibody titers were evaluated

using the ID Screen® Influenza H5 Indirect ELISA (product code = FLUH5S, according to the manufacturer instructions for use ver 1221) and the ID Screen® Influenza A Nucleoprotein Indirect (product code = FLUNPS, according to the manufacturer instructions for use ver 0416). In parallel, CEVA PHYLAXIA also performed the HI test using the HPAIV H5N8 strain (A/Belgium/chicken/U1700807 PTL807/2017). Animals were bled at 55 weeks and 57 weeks, i.e. 2 weeks after challenge, respectively. Mean, minimum and maximum titers as well as the CV% were calculated.

Monitoring of birds vaccinated with H5 RNA vaccine – SIENSANO RESULTS

SPF chickens were vaccinated at day 1 and at 4 weeks of age with a H5 RNA vaccine (from CEVA Santé Animale). The animals were challenged at 6 weeks of age by a strain H5N8. Antibody titers were evaluated using the **I**D Screen® Influenza H5 Indirect ELISA (product code = FLUH5S, according to the manufacturer instructions for use ver 1221) and the ID Screen® Influenza A Nucleoprotein Indirect (product code = FLUNPS, according to the manufacturer instructions for use ver 0416). In parallel, HI testing was also performed using the HPAIV H5N8 strain (Belgium 2017 A/Brahma chicken/Belgium/6153/2017). Animals were bled at 3, 4, 5, 6 and 8 weeks of age. Mean, minimum and maximum titers as well as the CV% were calculated.

Monitoring of birds vaccinated with Volvac® B.E.S.T. AI+ND vaccine

Layer and broiler flocks (n=55) from Egypt for a total of 980 samples were vaccinated between 6 and 14 days of age with Volvac® B.E.S.T. AI+ND vaccine from Boehringer Ingelheim. Animals were bled at 19 to 66 days post vaccination.

All the flocks were first tested with the ID Screen® Influenza H5 Indirect ELISA (FLUH5S) and the ID Screen® Influenza A Nucleoprotein Indirect ELISA (FLUNPS) kits. Two distinct groups have been identified: the unchallenged flocks and the challenged flocks. A preliminary baseline was calculated on the unchallenged flocks. Then results obtained from the challenged flocks were interpreted using the previously defined baseline and the known zootechnical information.

RESULTS

Specificity study of the FLUH5S kit

GD Deventer and FLI serum sample panel belonging to the H6, H7 and H9 subtypes were detected as negative. Samples belonging from H5 subtypes (H5N2 and H5N3) were detected as positive.

A total of 420 samples (320 sera from SPF birds and 100 sera from disease-free broilers) were tested to assess specificity. All samples were detected as negative.

Measured specificity with the FLUH5S kit = 100% (CI95%: 99,09%-100%), n=420

Monitoring of birds vaccinated with Vectormune AI (rHVT-AI(H5))

Samples from commercial layer-type chicks vaccinated with Vectormune AI rHVT-AI(H5) were tested with FLUH5S and FLUNPS kits as well as with the HI test, as explained above.

This FLUH5S kit has a good correlation with the HI test and it provides a very good detection of the antibodies developed after vaccination with a rHVT-H5 vaccination. The FLUNPS kit is a very good tool to be used as a part of a DIVA strategy: only challenged birds are detected with this kit while vaccinated animals are monitored using the FLUH5S kit.

Monitoring of birds vaccinated with H5 RNA vaccine – SIENSANO RESULTS

Samples from SPF chickens vaccinated with H5-RNA vaccine were tested with the FLUH5S and FLUNPS kits as well as with the HI test as explained above. The FLUH5S kit has a good correlation with the HI test and it provides a very good detection of the antibodies developed after vaccination with a H5 RNA vaccine. The FLUNPS kit is a very good tool to be used as a part of a DIVA strategy: only challenged birds are detected with this kit while vaccinated animals are monitored using the FLUH5S kit

.**Monitoring of birds vaccinated with Volvac® B.E.S.T. AI+ND vaccine**

Broiler and layer flocks vaccinated with Volvac® B.E.S.T. AI+ND vaccine were tested with the FLUH5S, FLUNPS.

The FLUH5S kit detects very well the antibodies developed after vaccination with Volvac® B.E.S.T. AI+ND vaccine. The FLUNPS kit is a very good tool to be used as a part of a DIVA strategy: only challenged birds are detected with this kit while vaccinated animals are monitored using the FLUH5S kit.

DISCUSSION

Specificity study of the FLUH5S kit

Results show that the FLUH5S kit is very specific to the H5 hemagglutinin subtype. The other subtypes are not detected with this kit, i.e. there is no cross-reactions with other AI subtypes.
In addition, the specificity was tested using the FLUH5S kit and all the samples coming from unvaccinated birds were negative with the FLUH5S kit.

Monitoring of birds vaccinated with Vectormune AI (rHVT-AI(H5))

Birds were vaccinated with the recombinant vaccine mentioned above. The results show that the FLUH5S kit is able to detect the antibodies developed after the vaccination. Indeed, for the layers at 55 and 57 weeks of age, mean titers of approximately 15 000 are obtained (the cut-off of the kit is 732).

The FLUNPS kit can also be used as a part of DIVA strategy: in the birds challenged at 55 weeks of age, antibodies developed due to the infection are detectable with this kit 2 weeks later (at 57 weeks of age). Indeed, the mean titer with FLUNPS kit at 57 weeks of age is approximately 4 000 (the cut-off of the kit is 668).
Finally, the results also show that the FLUH5S kit enables the monitoring of vaccination with a good correlation with the HI test.

Monitoring of birds vaccinated H5 (RNA vaccine) – SIENSANO RESULTS

Birds were vaccinated at day 1 of age and at 4 weeks of age with the H5 RNA vaccine. The results clearly show that the FLUH5S kit is able to detect the post-vaccination antibodies (the antibodies are even detected 1 week after the vaccination at 4 weeks of age). The mean titers obtained with the FLUH5S kit are around 8 000 at 5 weeks of age and around 10 000 and more at 6 and 8 weeks of age.

In addition, the FLUNPS kit can also be used as a part of a DIVA strategy: in the group of birds challenged at 6 weeks of age, we observed an increase at 8 weeks of age (2 weeks after the challenge). The mean titer is approximately 700 with the FLUNPS kit (cut-off = 668).

As before, the results also show that the FLUH5S kit enable to monitor the RNA vaccination with a good correlation with the HI test.

Monitoring of birds vaccinated with Volvac® B.E.S.T. AI+ND vaccine

All the flocks were first tested with the ID Screen® Influenza H5 Indirect ELISA (FLUH5S) and the ID Screen® Influenza A Nucleoprotein Indirect ELISA (FLUNPS) kits. Two distinct groups have been identified:

- The unchallenged flocks: all the flocks were found negative with FLUNPS kit.
- The challenged flocks: all the flocks were found positive with FLUNPS kit.

A preliminary baseline was calculated based on the unchallenged flocks. It is defined for a mean titer between 2 000 and 5 000. As for the challenge flocks, they were first tested with the FLUH5S kit. The previously calculated baseline was maintained. Some flocks obtained a mean titer higher than 5 000, suggesting a suspicion of H5.
As before, the results also show that the FLUH5S kit enable to monitor the Volvac® B.E.S.T. AI+ND vaccination.

CONCLUSIONS

The FLUH5S ELISA kit is based on a recombinant H5 protein. The results presented show that this new tool is very specific for the H5 hemagglutinin subtype. This FLUH5S kit has a good correlation with the HI test and it provides a very good detection of the antibodies developed after vaccination with a rHVT-H5 vaccination or with an H5 RNA vaccine or with Volvac® B.E.S.T. AI+ND vaccination.

In addition, the FLUNPS kit is a very good tool to be used as a part of a DIVA strategy: only challenged birds are detected with this kit while vaccinated animals are monitored using the FLUH5S kit.

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INSIGHTS ON DAY OLD BROILER IBV VACCINE TAKES

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INTRODUCTION

Day of age vaccination is one of the most convenient ways of immunizing broiler chickens against respiratory diseases. While this is a common practice, different delivery options are available. Water base spray cabinets and gel drop cabinets are the most widely used. While spray cabinet vaccine application has been extensively researched (1) (2) and calibrated to volumes that increase vaccine takes in hatcheries (3), some producers opt to use gel drop cabinets due to the delivery of salmonella and coccidia vaccines at the same time IBV is delivered. These systems use a gel applicator bands that release gel drops on top of the chickens while the chicken box passes through a belt. These drops are ingested or inhaled while chickens preen themselves. New investigations discuss the differences of spray and gel IBV vaccination using Mass and 793B IBV genotypes in broiler chickens (4). The results showed that Mass type persisted longer in gel vaccinated chickens. In addition, gel administration was equally effective and less hard on chick temperature. The current project focuses on understanding the effectiveness of both vaccination methods measuring vaccine takes at 4 days post application and differences observed when takes from gel vaccination are measured at day of vaccination compared with 3 days post vaccination.

MATERIALS AND METHODS

The first trial focused on the comparison between spray and gel vaccination takes. For that we used samples collected in the field 4 days post gel or spray vaccination.

In the second trial, choanal swabs were collected from birds two hours after gel vaccination at the hatchery and 3 days post vaccination. RNA was extracted from all samples and IB virus particles load were calculated based on RT-qPCR detecting the M gene (5). Viral load results were compared using one way ANOVA using Prism Graph pad.

RESULTS AND DISCUSSION

The first trial focuses on the "takes" of birds vaccinated against IBV. We compared viral particles obtained after RT-qPCR's. Half of the swabs were obtained from birds vaccinated with IBV via spray cabinet and the other half vaccinated via gel drop cabinet. All birds belonged to the same ranch.

As shown in figure 1, birds vaccinated via gel had a significantly better takes compared with birds vaccinated via spray cabinet. These results agree with what was reported by Legnardi (4). Both assessments were done 4 days post vaccination. While 4 days of age seems an adequate time for the virus to spread in the flock, it seems a long time to measure takes and might reflect more rolling reaction than shedding. Rolling reaction contributes to viral evolution and variant generation (6). Reason why we decided to investigate gel vaccination takes two hours and three days post vaccination.

When we investigated gel application at different times, numerical differences were noticed between the two vaccination lines at day of age, no statistical differences were detected. This numerical difference increased when viral load was measured 3 days post vaccination. House 14 had an increased viral load than house 13. These results emphasize the importance of a proper vaccination at day of age and how takes should be measured after application and at the field. Better takes at day of age reflect on better viral distribution in the flock and consequently better immunization. While we did not measure protection, we assume that better loads will indicate better immune responses and better protection. This should be studied to adequately correlate takes and protection.

Our experiment was able to confirm the good takes delivered by gel vaccination at day of age. In addition, raises the concern that vaccine takes should be measured hours and not days after vaccination since virus variation and evolution might have a role in generation of new variants.

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Figure 1. Comparison of the viral load in choanal swabs obtained from birds' gel and spray IBV vaccinated at four days post vaccination.

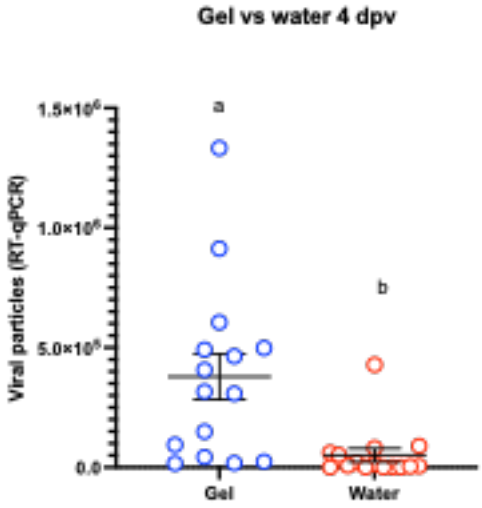
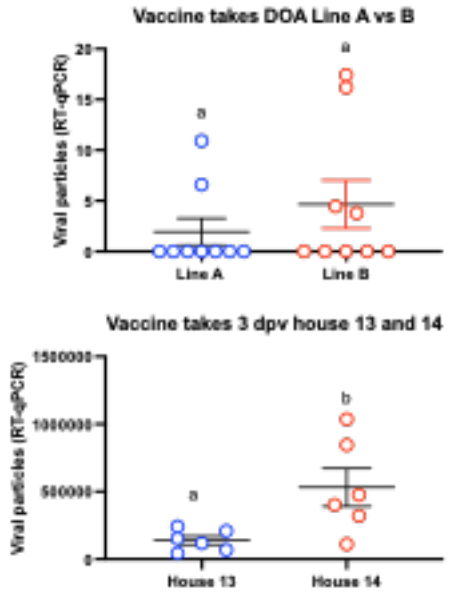


Figure 2. Viral load from swabs collected from gel IBV vaccinated birds two hours and three days post vaccination. Different vaccination lines were compared.



CLOSTRIDIUM PERFRINGENS: NEW INSIGHTS INTO THE EPIDEMIOLOGY OF THIS PATHOGEN IN BROILER CHICKENS

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INTRODUCTION

Since the ban on the use of antibiotic growth promoters in food producing animals by the European countries at the end of the 1980s, Clostridium perfringens, through the necrotic form of the enteritis it causes in commercial broiler chickens, emerged as one of the biggest threats challenging the long-term sustainability of the poultry industry (1). Consequently, the scientific community has increasingly undertaken efforts to better understand necrotic enteritis and C. perfringens. While all the attention was focused on the animal pathogen aspect of this bacterium, its role as a zoonotic pathogen was overlooked. Zoonotic C. perfringens is also known as enterotoxigenic C. perfringens due to the enterotoxin encoded by the cpe gene it uses as a virulence factor to cause gastro-intestinal diseases in humans (2). More than 1 million cases of foodborne disease illnesses are reported in the U.S each year (3). Even though meat, including poultry products, and fresh produce are recognized as the main sources of enterotoxigenic C. perfringens exposure for humans, these reservoirs and their associated transmission routes remain poorly described, as do the genetic features of the C. perfringens strains circulating through this network (2, 4, 5, 6, 7, 8). Even though C. perfringens is recognized as a normal inhabitant of the poultry intestine and is routinely found on broiler chicken farms and at the slaughter plant level, very few studies have looked into the presence of the zoonotic sub-population of this pathogen in these environments (9, 10, 11). After revealing that 21% of the broiler chicken flocks entering two different slaughter plants in Quebec and that up to 25% of their associated air-chilled carcasses were positive for the presence of C. perfringens cpe gene, our research group evaluated the role of the broiler farm as a reservoir of enterotoxigenic C. perfringens (12).

MATERIALS AND METHODS

Farm selection and sample collection. Broiler chicken farms in Quebec were selected based on a voluntary participation basis. Two visits were planned during a same rearing period: just before chick placement and prior to catching, at time of slaughter. Samples were collected during each visit, except for the chick boxes and the transport coops that were sampled at chick placement and before catching, respectively. Environmental surfaces (100 cm2) including walls, fans, feeders, chick boxes and transport coops were sampled using a swab. The litter was sampled using boot swabs that were worn during the visit, and feed was collected directly from the grain silo on the farm, outside the barn. Detection of C. perfringens enterotoxin-encoding gene. All samples were submitted to i) a pre-enrichment step in fluid thioglycolate enrichment broth, ii) total DNA extraction using The InstaGene matrix DNA extraction protocol with a 10% Chelex 100 solution in water, and iii) a PCR approach to confirm the presence of cpe.

RESULTS

Farm selection and sample collection. A total of 30 broiler chicken farms were visited during a same rearing period, on the day of chick placement and before catching, at time of slaughter. During each visit, ten environmental samples of 100 cm2 surfaces were collected from each of the wall, fan, feeder, chick boxes and transport coops surfaces, a boot swab was used for litter sampling and a sample of approximately 250 grams of feed was recovered, for a total of 360 samples. Detection of C. perfringens enterotoxin-encoding gene, cpe. Based on the PCR detection of cpe, 6.5% of the chick boxes and 0% of the transport coops were found positive for the presence of enterotoxigenic C. perfringens. While 16% of feed sample were identified as cpe-positive at the end of the rearing period, 11% of these samples collected prior to chick placement were found positive for the presence of this gene. Regarding the rearing environment, 0%, 3% and 0% of the wall, fan and feeder surfaces sampled prior to chick placement were identified as cpe-positive, respectively, whereas these surfaces were attributed this same status for 3%, 3% and 7% of the samples collected at time of catching, respectively. The litter sampling revealed the presence of cpe for 6% and 7% of the broiler chicken farms sampled at the beginning and at the end of the rearing period, respectively.

CONCLUSION

In the perspective of adopting a One Health approach that aims to enhance the long-term health of people and their coexistence with healthy animals and ecosystems, and while, as a consequence, the world largest broiler chicken producing countries are relying less and less on the action of antibiotics to keep necrotic enteritis and C. perfringens under control, results from this study conducted by our group highlight the urgent action needed to support more extensive research on the epidemiology and molecular biology of enterotoxigenic C. perfringens from poultry origin, as well as on control strategies aligned with this One Health concept. A first step could then be to isolate and characterize enterotoxigenic C. perfringens along the broiler chicken production chain in order to better estimate the risk associated with its presence in poultry.

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ONLINE DISCUSSION OF RESPIRATORY DISEASE SYMPTOMS IN BACKYARD POULTRY FOR EARLY DISEASE DETECTION

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SUMMARY

Over the past decade, several outbreaks of poultry diseases with respiratory clinical signs have resulted in significant damage to the poultry industry and resulted in significant depopulation of commercial and backyard poultry (BYP). These include two outbreaks of Avian Influenza and one outbreak of virulent Newcastle Disease, both of which often present first in BYP in California before infecting commercial flocks.

Detection of these diseases in non-commercial reservoirs relies on BYP owners submitting dead or sick birds to diagnostic labs (i.e. passive surveillance), which results in an inefficient surveillance system. Self-reported symptoms of human diseases such as COVID-19 on social media have shown to be positively correlated with and predictive of increases in case counts. Here we explore leveraging BYP owner reports of respiratory symptoms on forums and social media for as a complementary tool for early disease detection.

INTRODUCTION

Rapid detection of cases of infectious disease during an outbreak is essential for effective containment, particularly when the causal organism is highly virulent and/or infectious. The current system of disease detection in poultry relies on submission to diagnostic laboratories. This system inherently includes biases and delays in detection due to various factors including proximity to diagnostic labs, ability to pay associated laboratory fees, and associated delays in diagnostics. In humane medicine, self-reported clinical signs on social media are used to predict new cases of disease. Specifically, reports of symptoms and diagnoses of COVID-19 have been shown to be predictive of daily case counts up two weeks ahead of official reports (1). In this study, we explore posts on a backyard chicken forum discussing disease for trends in discussion of clinical signs of respiratory disease. Ultimately, these data may be useful in constructing detection models for early warning of poultry disease outbreaks at a spatio-temporal level.

MATERIALS AND METHODS

An online forum centered around discussion of illnesses and injuries in backyard poultry with daily users was identified. All posts from January 1st 2018 to October 18th 2022 containing discussion of clinical signs associated with common respiratory diseases of poultry were cataloged. At least one of the following terms had to be present for collection: "gasping", "coughing", "sneezing", "rales", "tremors", "paralyzed limbs", "twisted neck", "circling", "clonic spams", "paralysis", "torticollis", "wry-neck", "stargazing", "depression", "watery green diarrhea", "swelling head", "swelling neck", "not laying", "ocular discharge", "swollen infraorbital sinuses", "sinusitis", "decreased egg production", "cyanosis", "turning blue", "edema", "swelling", "bloody oral discharge", "nasal discharge", "mucus", "boogers", "green diarrhea", "opisthotonos", "standing weird", "droopy wings", "incoordination", "stumbling", "tripping", "drooping wings", "tracheal rales", "wheezing", "bubbling", "Conjunctivitis", "dyspnea", "breathing heavily", "facial swelling", "depressions", "huddling", "reduced feed consumption", "weight gain", "depression", "ruffled feathers", "wet droppings", "greater water intake", "misshapen eggs", "malformed eggs", "gasp", "cough", "circles", "died", "death", "dying", "die", "killed", "kill".

Descriptive statistics and comparison with the timeline of recent outbreaks of Newcastle disease (ND) and highly pathogenic avian influenza (HPAI) were performed in Excel. Posts were tagged as ND, HPAI by searching for terms specifically mentioning each disease using regular expressions in Python. Similarly, posts were tagged as having neurological signs, respiratory signs, or discussion of mortality using relevant terms from the above list of terms (Table 1).

RESULTS

A total of 208,519 posts from 26,387 discussion threads were collected over the study period. Graphing the data by month shows seasonality with year peaks observed in mid spring to early summer and yearly troughs in late autumn

(Figure 1). The year with the highest monthly average of posts was 2020 (4,064 posts) while 2022 had the lowest monthly average (2,992 posts). After the regular expression tags were applied, 747 posts specifically mentioned ND while 12,737 posts discussed AI. Discussion of ND was higher during the 2018 to 2020 outbreak period compared to the period after the California Department of Food and Agriculture (CDFA) declared ND eradicated from California (2), and there was a small increase in volume of discussion of HPAI from March 2022 to May 2022 during the 2022 HPAI outbreak compared to previous years (Figure 2).

Mortality (n=38,651) was discussed more than respiratory (n=32,027) or neurological signs (n=3,976) (figure 3). On average, 666 posts per month discussed mortality, 552 posts discussed respiratory signs, and only 69 posts per month discussed neurological signs. The trend of each of these discussion topics generally followed the same seasonality as the overall volume of posts, but neurological signs discussions had a lower coefficient of variance when compared to discussion of respiratory signs and mortality (neurological cv=25.3%, respiratory cv=27.1%, mortality cv=27.3%). Discussions of respiratory clinical signs and mortality were increased throughout 2018, January to May 2019, and April to July 2020 when compared to 2021 and 2022.

DISCUSSION

This initial analysis of the temporal trends of discussions of clinical signs associated with respiratory diseases of poultry show a potential relationship to concurrent disease outbreaks. Discussion of ND as well as respiratory signs and mortality increased during key points during the 2018-2020 ND outbreak. Additionally, there were increased discussion of ND and related symptoms before the outbreak was declared in May of 2018 (2). These data also provide insight into the seasonality of discussions of disease in online BYP forums. Peak discussions occur annually during the spring and early summer, which contradicts late autumn and winter peaks of causes of respiratory poultry disease (3, 4). This suggests that trends in discussion of clinical signs may be driven by factors other than disease prevalence. Spring hatches and less frequent health assessments during cold months should be considered as factors in seasonality for further analysis.

Though trends in ND discussion aligned with key events during the 2018-2020 ND outbreak, discussion of HPAI only increased slightly during the peak period of the current 2022 HPAI outbreak from March to May of 2022 (5). Discussions of mortality and respiratory signs during this period also peaked but were still much lower than during previous years. It is possible that the increases in discussion of these diseases during peak periods of disease outbreaks are due to heightened awareness of the disease due to mainstream media coverage (6).

Discussions of neurological signs were an order of magnitude lower than discussions of mortality or respiratory signs throughout the study period. While this may be due to significantly lower incidence of diseases with neurological presentations, it is possible that BYP owners do not have adequate resources to recognize neurological issues in their birds. Further exploration of this data and outside studies should be considered to measure BYP owners' understanding of deviations from normal motor control and behaviors. This may be an area of extension that needs improvement.
While this study has identified seasonality of BYP forum discussions of poultry diseases and peaks in relation to recent respiratory disease outbreaks, further investigation is needed to determine if a predictive model can be created with this data. While case counts and locations are readily available for the current HPAI outbreak, further case data from the 2018 to 2020 ND outbreak is needed to determine if discussions of clinical signs relevant to ND precede new cases. Self-reported user location data may also allow for spatial analysis of posts discussing regional outbreaks like ND in Southern California. Since some of the terms used for cataloging posts were broad and not unique to respiratory disease, further text analysis is needed to include only discussions of infectious disease. This can be achieved by using key word extraction and other natural language processing techniques to identify the topics of each post. Once topics are extracted, Boolean logic and regular expressions can be used to define specific combinations of words (e.g. "cough" OR "wheeze" OR "wry-neck" AND "dead") that are more closely associated with infectious respiratory disease. A statistical model relating these data and case counts and locations of recent disease outbreaks may then describe the temporal relationship between new cases and discussion of clinical signs.

While traditional passive surveillance is an important tool in the identification of infectious diseases in non-commercial poultry, limitations in this type of passive surveillance reduce the time to detection. Using text analysis, natural language processing and mapping offer a novel complementary approach which stakeholders could use to identify potential outbreaks before traditional methods further clarify the infectious agent.

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Table 1. Terms included in regular expressions to assign tags. The regular expression used detects the bellow patterns inside of other words. For example, “gasp” would also detect “gasping” since the former exists in its entirety inside the latter.

Tag Name	Terms
ND Tag	'newcastle', 'vnd'
HPAI Tag	'flu', 'influenza', 'HPAI'
Neurological Tag	'spasm', 'twisted neck', 'circling', 'paralysis', 'torticollis', 'wry-neck', 'stargazing', 'incoordination', 'tripping', 'stumbling', 'drooping wings', 'tremors', 'paralyzed'
Respiratory Tag	'sneezing', 'rales', 'swelling', 'swollen', 'wheezing', 'gasp', 'cough', 'sneeze', 'wheeze', 'breathing'
Mortality tag	'death', 'dying', 'die', 'kill'

Figure 1. Total volume of forum posts collected by month. Seasonality is noted, with annual peaks in late spring and early summer and troughs in late autumn and winter.

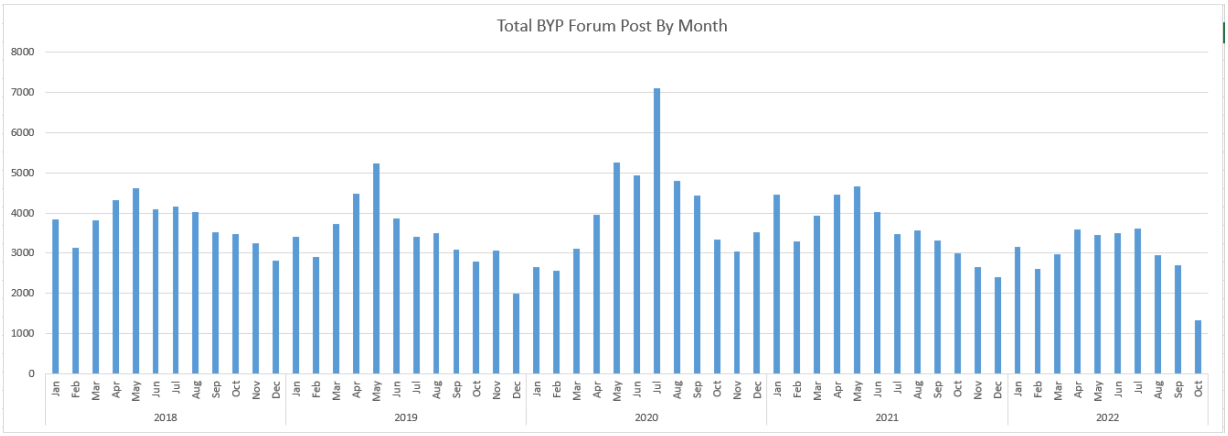


Figure 2. Volume of posts discussing Newcastle Disease and Highly Pathogenic Avian Influenza.

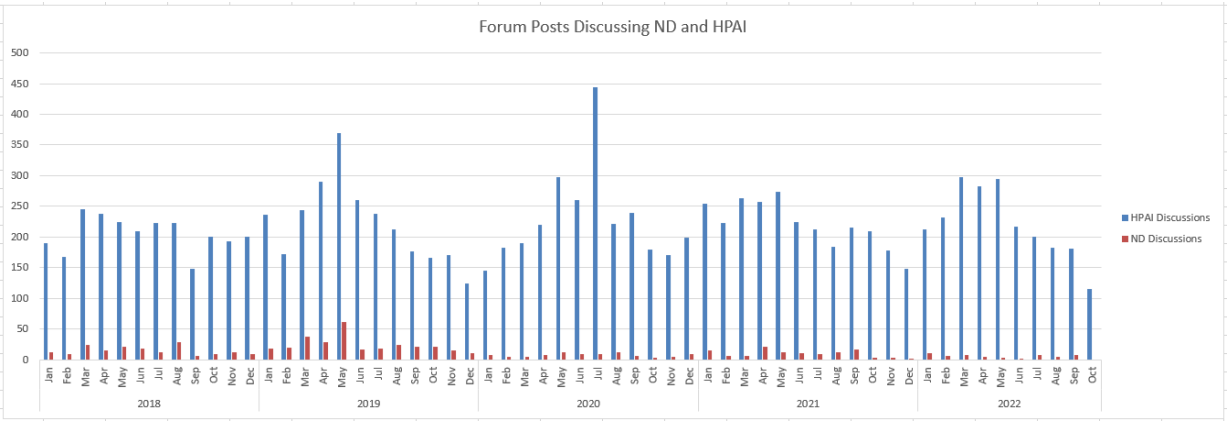
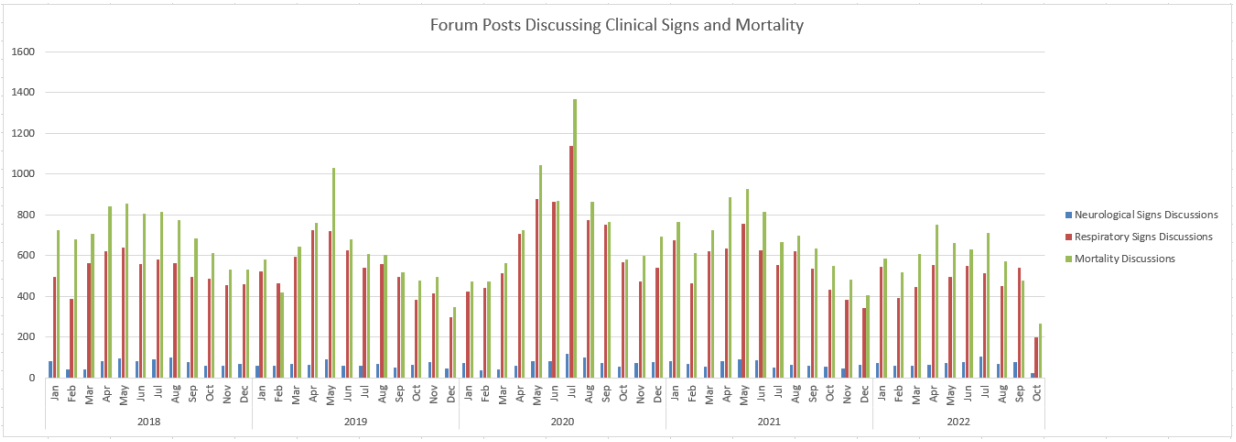


Figure 3. Volume of posts discussing clinical signs and mortality. Discussion of mortality and respiratory clinical signs are markedly increased in 2018 and early 2019. The volume of discussion of neurological signs is an order of magnitude less than that of respiratory signs and mortality.



TEMPORAL TRENDS AND SPATIAL CLUSTERING OF NON-COMMERCIAL POULTRY SALES IN CALIFORNIA FOR MODELING VIRULENT NEWCASTLE DISEASE OUTBREAKS

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SUMMARY

Backyard poultry play a significant role in the spread and persistence of infectious disease outbreaks in California, as evidenced by the 2002 and 2018 outbreaks of virulent Newcastle disease and the 2022 outbreak of H5N1 avian influenza. The close proximity of backyard flocks to each other and to commercial poultry facilities as well as sub-optimal biosecurity and vaccination practices poses a significant risk to the California poultry industry and beyond due to the realities of commerce and supply chains. A lack of understanding of spatio-temporal distributions of backyard birds throughout the state make targeted outreach and accurate disease modeling challenging. Here we examine data collected from online poultry sales advertisements over the course of a year to determine temporal trends in sales and spatial clustering of sellers. We leverage this data to inform disease models investigating the efficacy of vaccination programs for vND.

INTRODUCTION

Backyard poultry (BYP) and gamefowl (GF), collectively non-commercial poultry (NCP) have been linked to the transmission of infectious diseases in the United States, including outbreaks of Newcastle disease (ND) in 2003 (1) and 2018 and Salmonella Hadar and Salmonella Enteritidis in 2021 (2). Since NCP are an engrained aspect of life in California (3,4) and urban BYP ownership is an increasing trend in the United States (5), understanding spatial and temporal distributions of NCP is necessary for effective disease modeling and outbreak response. Traditional survey methods are limited by reach, survey frequency, and trust in government regulators and academics, which introduce various biases in the ultimate response rate (6). COVID-19 and the current highly pathogenic avian influenza outbreak has also been shown to reduce survey responses due to fewer in person interaction within social networks (7). A lack of enforced registration policy in the United States further inhibits the ability to understand locations of NCP. Therefore, more robust methods of surveillance that do not rely on in- person interaction or additional action on the part of BYP owners are necessary.

The field of computational social science centers around investigating social phenomena through big data and computational methods, often employing the "digital footprint" of online social activities. In this study, we apply these principles to epidemiological investigation by analyzing the spatial and temporal characteristics of online NCP sales on a classified advertisement website in several regions of California. These data present a novel source of information for disease modeling and outbreak control.

MATERIALS AND METHODS

Data collection. A classified advertisement site was identified as a significant platform for private party bird sales after an initial broad internet search. Posts selling birds on this site were cataloged on a weekly basis from November 9th 2021 to November 2nd 2022 for the following regions: Los Angeles County, San Diego County, Imperial County, and the Inland Empire (comprising Riverside and San Bernardino Counties). Throughout the collection period, additional regions were added to the monitoring list (fig 1). Post data was collected if the post title or description contained any of the following match terms and did not include any of the following ignore terms:

Match terms. "chicken", "rooster", "hen", "bird", "pigeon", "egg", "quail", "duck", "goose", "chick", "pullet", "gallos", "gallina", "huevo", "pato", "pavo", "guajolote", "codorniz", "turkey", "aseel", "asil", "scandaroon", "rhode"

Ignore Terms:"cage", "wire", "coop", "trailer"

The url, title, description, reported location, post date, and later price were collected for each post. Each post was assigned the following topic classifiers based upon inclusion of the associated terms in the title or description: Egg {"egg", "fertilized", "dozen", "huevo"}; Chicken {"chicken", "rooster", "hen", "pullet", "gallina"};

Pigeon{“pigeon”}; Rooster {“rooster”, “cockerel”, “gallo”}; Other Species{ "quail", "duck", "goose", "pavo", "pato", "guajolote", "turkey", "codorniz"}.

Data cleaning. Data cleaning was performed using Python (8) and the Pandas library (9) v1.1.3. The title field for each post was manually checked for key terms that corresponded to irrelevant posts (e.g. “bird” followed by “bath” or “cage”). The key terms were added to a list, which was then used to create a regex pattern. The title fields of each post were checked for the key terms and removed. This process was repeated until no false positives remained in the dataset.

Data analysis. After cleaning, data was exported to a Microsoft Excel worksheet file. Descriptive statistics were performed in Excel 2016 (10). Unique posts were defined by removing duplicate uniform resource locators (URLs). Unique posts in southern California (Inland Empire, Los Angeles County, and San Diego County) were stratified by month and city/CPD and a Kruskal-Willis test was performed to determine if significant temporal trends exist. Since the data was significantly right skewed, this test was performed using only cities with at least 6 observations and again with the top 25 cities. Using data extrapolated from the 2018-2020 ND positives map provided by CDFA (11), southern California cities were categorized as either ND positive or negative, and a Kruskal-Willis test was performed to determine if a difference in the number of posts in ND positive location and ND negative locations existed. All statistical tests were performed in R(12). Mapping was performed in ArcGIS Pro (13) using the California Board of Equalization (CBE) 2022 dataset (14). The total number of unique posts for each municipality and census designated place were spatially joined with the CBE data to create a choropleth map.

RESULTS

The total number of posts collected across all regions monitored was 42,544, and 17,613 unique posts were identified. The Inland Empire (Riverside and San Bernardino Counties), Los Angeles County, San Diego County, and the Sacramento area (bounded by the San Francisco Bay Area to the west, El Dorado and Placer Counties to the east, the Stockton area to the South, and the Yuba-Sutter area to the North) had the highest average monthly posts by a margin of 185 posts (Table 1).

During the course of the study, 572 cities or CDPs throughout the state had at least one advertisement selling birds. The five locations with the most posts per month were Sacramento (84.5 posts), Vista (63.1 posts), La Mesa (62.7 posts), Fresno (56.8 posts), and Perris (52.7 posts) (Figure 1). The data is heavily right skewed (skewness = 5.14, kurtosis = 35.8), with 92.1% of locations having less than 10 posts per month, and 48.1% of locations having 1 or less posts per month. Of the 44 locations that had 10 or more posts per month, 15 locations were in the Inland Empire while Los Angeles County, San Diego County, and Sacramento County each had 6 locations. When stratified by month, there was no significant difference in the number of advertisements posted ($p = 0.883$ for locations with at least 6 observations, $p = 0.702$ for top 25 locations). Of the 30 cities with ND positives during the 2018 to 2020 outbreak, 12 had over 100 posts during the study period. Among these cities were Perris, Riverside, Hesperia, and Los Angeles. Post count was shown to be a significant factor ($p<0.001$) when comparing cities with and without ND positives.

Mapping total advertisement counts shows sales hotspots in ND affected areas (Figure 2). The western Inland Empire shows highly clustered cities with over 100 posts that overlap areas with the majority of ND cases, including Norco, Perris, and Riverside. Northern San Diego County also contains several high post volume locations, which coincide with the single ND positive in San Diego during the last outbreak. Hotspots in the Central Valley tend to be more concentrated around major cities.

DISCUSSION

The results of mapping the distribution of online bird sales throughout Southern California and the Central Valley as well as comparing advertisement counts with ND positives allows for a better understanding of the overall distribution of NCP in the state. The lack of seasonality in these data suggests that the distribution of NCP may remain consistent throughout the year, though more data throughout the state needs to be collected over multiple years to confirm this. Stratifying the advertisements by the type of birds being sold (i.e. laying hens, gamefowl, other species) may yield differences in sales as a function of seasonality. Although a correlation between the number of posts in a location and locations of ND positives was detected, ND positive data was approximated from maps with low spatial resolution, and actual ND location data should be used to confirm this relationship.

The Sacramento and Fresno areas were the only areas outside of Southern California with a comparable number of advertisements as ND affected areas. While there is less spatial overlap between NCP and commercial poultry in these regions, it is important to interpret the data presented as “out-going” in the context of disease spread risk. That

is, it is unknown where the NCP being sold in these high population density areas are being brought, and they may be moving out of cities to areas with more commercial poultry. Knowing that these areas contain large populations of NCP is important if ND or a disease with a similar manner of spread reaches the northern Central Valley.

These sales data provide a novel source of NCP distribution information that supplements the outdated survey data available. It is important to note that online data only capture sales by internet users and do not necessarily reflect local in person or live bird market sales. Still, the volume of data available represents a significant source of data for disease modeling and response preparation. Techniques like these have the potential to improve the overall accuracy of models which could positively impact the responses to a disease outbreak. In a future study, these data will be used to approximate BYP premises for SEIR modeling. Additionally, since these data represent the movement of birds, online sales platforms should be monitored during future outbreaks for detections of breaches of quarantine.

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Table 1. Monitoring periods and number of advertisements cataloged. The Inland Empire, Los Angeles County, San Diego County, and the Sacramento Area had significantly higher numbers of posts when compared to other regions.

Region	Start Date	End Date	Total Number of Unique Posts	Average Posts Per Month
Inland Empire	11/6/2021	11/2/2022	5745	477
Los Angeles County	11/6/2021	9/14/2022	3526	339
San Diego County	11/6/2021	11/2/2022	3986	331
Sacramento Area	6/22/2022	11/2/2022	1393	314
Fresno County	5/25/2022	11/2/2022	694	129
San Francisco Bay	7/13/2022	8/10/2022	103	110
Modesto Area	5/25/2022	11/2/2022	477	89
Visalia Area	6/1/2022	11/2/2022	326	64
Orange County	6/1/2022	11/2/2022	298	58
Stockton Area	6/15/2022	11/2/2022	224	48
Bakersfield Area	6/8/2022	11/2/2022	210	43
Ventura County	7/27/2022	11/2/2022	140	43
Merced County	6/29/2022	11/2/2022	130	31
Imperial County	11/6/2021	11/2/2022	339	28
Palm Springs Area	7/6/2022	9/7/2022	22	10
		Total	17613	141

Figure 1. Spatial distribution of advertisements selling birds during the study period. In addition to Southern California, the Fresno and Sacramento areas are hotspots for bird sales.

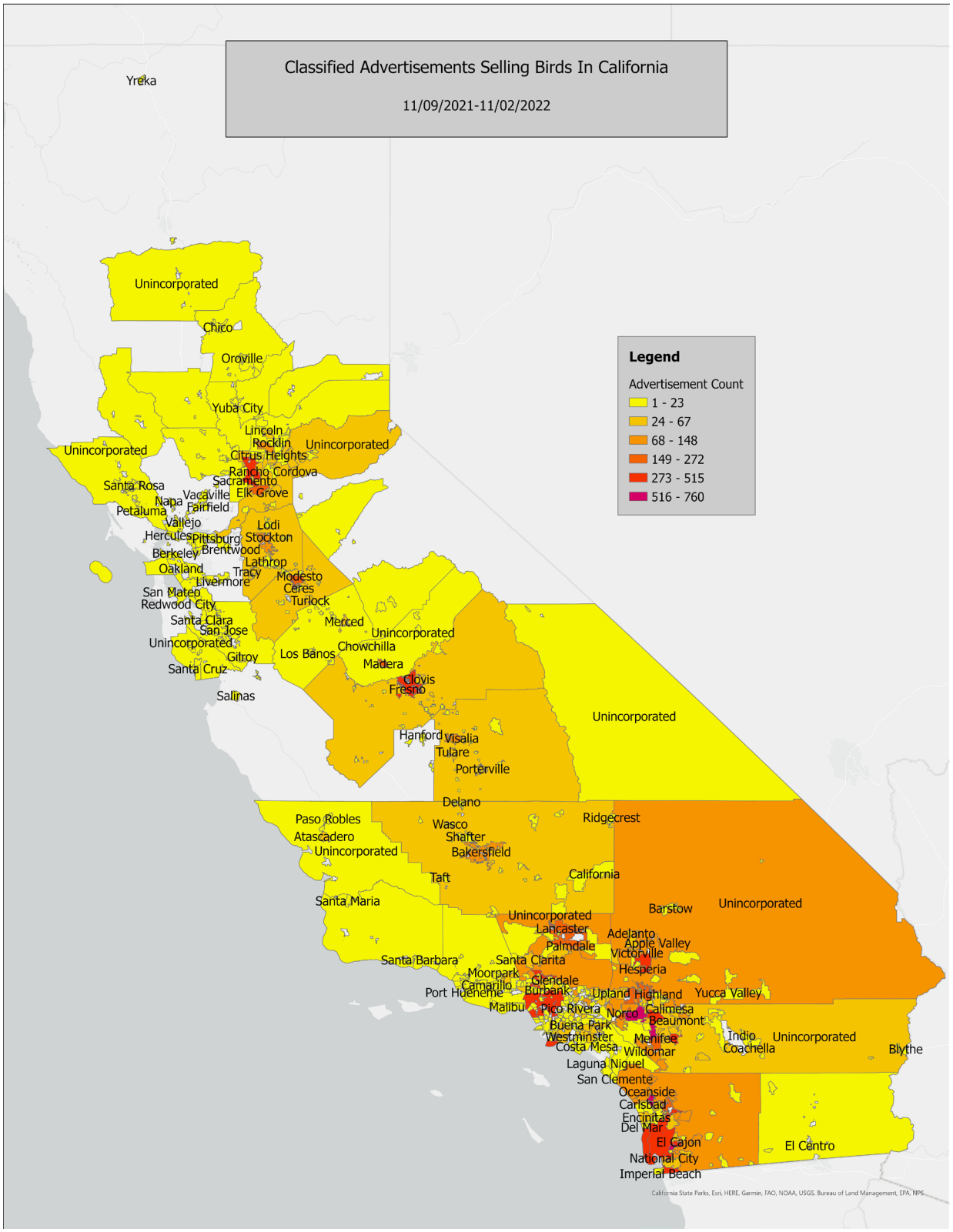
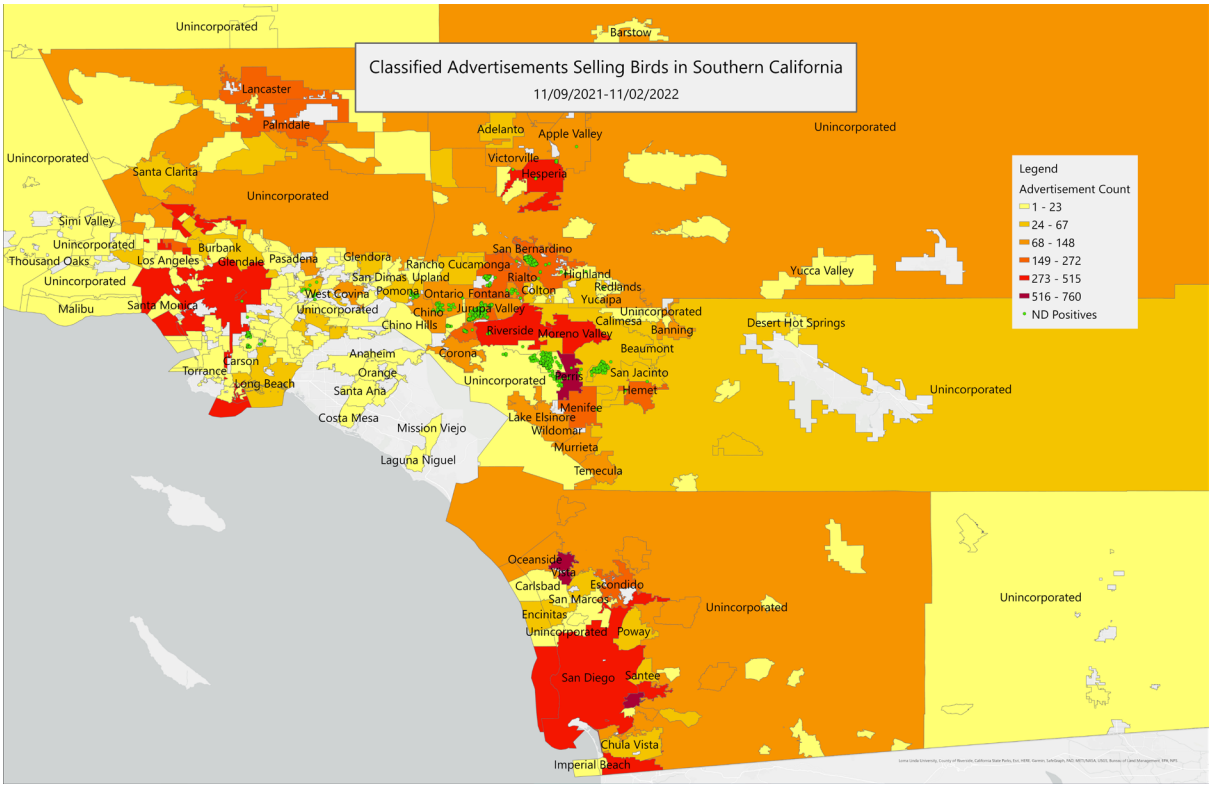


Figure 2. Advertisements selling birds in Southern California by city/CDP. High spatial overlap is observed between cities/CDPs with high bird sales and ND cases during the 2018-2020 outbreak.



USING THE WATERFOWL ALERT NETWORK TO UNDERSTAND WATERFOWL ROOSTING HABITAT IN CLOSE PROXIMITY TO COMMERCIAL POULTRY

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SUMMARY

The current outbreak of highly pathogenic avian influenza (HPAI) in North America has led to the depopulation of over 50 million poultry in 47 states with an economic impact of over $1 billion. Current prevention efforts by commercial producers focus primarily on structural and operational biosecurity. New and emerging remote sensing technologies have the capability to offer science-based information on movements of waterfowl, the primary reservoir of HPAI. The Waterfowl Alert Network (WFAN) is the first commercial service which offers daily predictions of waterfowl abundance at 250m granularity during the migration season. The technology builds statistical models that leverage remote sensing technologies including 160 Next Generation Weather Radar (NEXRAD), daily MODIS imaging and daily PRISM temperature readings. The WFAN ranks farms based on proximity to roosting waterfowl at different buffers (1-4km) and time periods (day, week, month). Several examples will be presented to demonstrate the efficacy of the tool.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) poses an existential threat to the poultry industry. Over 13% of North American laying hens were depopulated during 2022 due to HPAI along with significant populations of turkeys, broilers, breeders, and upland game birds (1). Migratory waterfowl carry and spread highly pathogenic avian influenza viruses during migration between breeding grounds and wintering sites (2). Novel surveillance methods are needed to understand how spatial distributions of waterfowl near commercial poultry relate to HPAI cases. The National Weather Service operates 160 Next Generation Weather Radar (NEXRAD) in the United States. The reflectivity product of these NEXRAD radar can be used to identify bird migration stopover sites (3). In this study, we employ a statistical model trained on NEXRAD data that predicts daily densities of waterfowl to investigate the spatial-temporal relationship between waterfowl densities and HPAI cases.

METHODS AND MATERIALS

Statistical model. A poultry company in the Midwest with over 300 farms ("company A") had several cases of HPAI during 2022. A study grid was created that covered all of company A's farms. Six NEXRAD radar were identified in the established study area. For each of these six NEXRAD radar, three years of daily data during the migration season (from November to March of the following year) were screened for contamination by precipitation or other artifacts at the 0.5° reflectivity angle (or closest available angle) in an 80km radius from the radar from one hour before civil twilight to one hour after. The high reflectivity values in the non-contaminated radar data represent waterfowl flying through the radar beam. These radar reflectivity data were then used to train a spatial model that predicts reflectivity due to birds across the study area. Several covariates were included in this model, including USDA NASS crop data, PRISM temperature and precipitation data (4), and MODIS imaging (5). The resultant model creates a daily spatial density prediction at 250mx250m resolution as soon as that days covariates are available. The statistical model is valid from November 1st to March 31st of the following year.

Farm surveillance and analysis. For each of company A's farms, the total predicted daily reflectivity was calculated at a 1km, 2km, 3km, and 4km radius around the farm from November 1st 2022 to February 4th 2023. During this study period, four of company A's farms tested positive for HPAI. Descriptive statistics and temporal analysis were performed in Excel.

RESULTS AND DISCUSSION

The average reflectivity of company A's farms was highest during the first half of November and sharply dropped off in the third week of November. Average reflectivity remained low through the remainder of the study period (figure 1). This is consistent with waterfowl migration patterns in the region (6). On the days HPAI was detected at the infected farms, farms 1, 3, and 4 had 4km reflectivity values close to the mean 4km reflectivity of all farms in the study area. Data was not available for farm 2 on the day of infection. However, farms 1, 2, and 3 had at least 3 4km daily reflectivity values greater than one standard deviation above the 4km daily mean (Table 1).

While these data do not show a "smoking gun" in terms of causality when comparing daily reflectivity values produced by the model and HPAI positives, it does suggest a relationship may exist. An increased sample size of HPAI positive farms as well as additional structural biosecurity context may yield a better understanding of HPAI infection risk to a farm due to waterfowl presence. A random forest model that takes biosecurity variables into account, including house ventilation type, house age, type of poultry, and other covariates along with bird reflectivity values can be used to help identify further relationships.

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Figure 1. Average reflectivity of all farms at 4km remains high from November 1st to November 11th, then drops sharply and remains low for the rest of the study period.

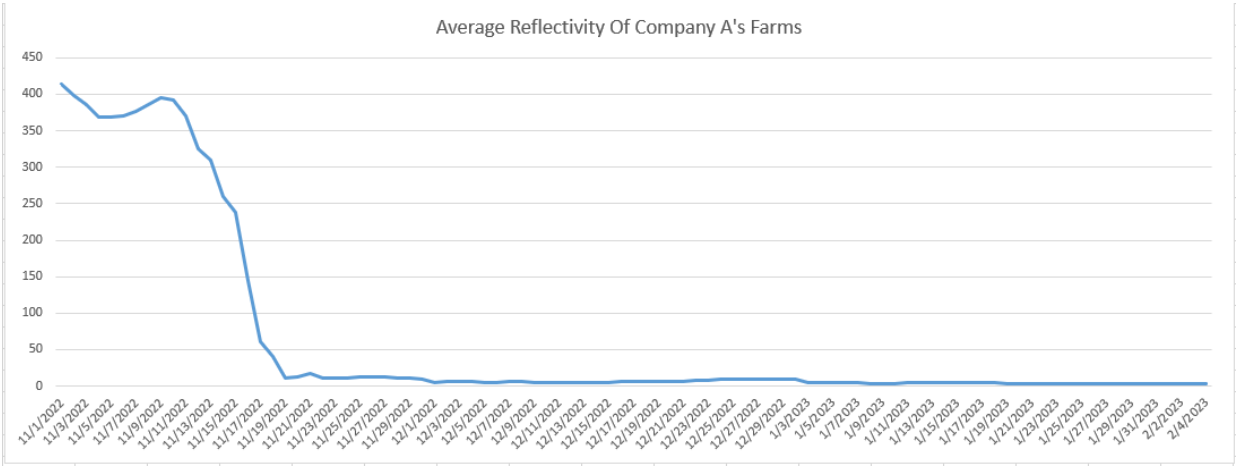

Table 1. Farms 1 through 4 in the table below tested positive for HPAI during the study period. Though no HPAI positive farms had 4km reflectivity values significantly above the daily mean at time of detection, farms 1, 2, and 3 had days with 4km reflectivity values greater than one standard deviation above the mean in the two weeks preceding infection. Bold numbers indicate reflectivity values greater than one standard deviation above the mean. X's indicate days outside of the study period.

Farm	Metric	i-14	i-13	i-12	i-11	i-10	i-9	i-8	i-7	i-6	i-5	i-4	i-3	i-2	i-1	Infection (i)
Farm 1	4km Reflectivity	x	x	**749**	527	**535**	**536**	**534**	**535**	**541**	**781**	363	353	342	336	368
	Mean 4km Reflectivity	x	x	413.6	398.3	385.9	368.8	368.7	369.8	376.9	386.5	395.4	391.7	370.9	325.3	309.4
	Standard Deviation	x	x	165.1	153.3	145.4	138.5	138.4	139.1	143.9	152.5	173.0	163.6	149.4	140.2	150.5
	1 STDEV Above Mean	x	x	578.7	551.5	531.3	507.3	507.1	508.9	520.8	539.0	568.4	555.2	520.3	465.5	459.9
Farm 2	4km Reflectivity	506	509	538	**730**	**718**	**538**	355	354	258	274	174				
	Mean 4km Reflectivity	369.8	376.9	386.5	395.4	391.7	370.9	325.3	309.4	259.7	238.3	145.2	61.0	40.4	10.9	13.0
	Standard Deviation	139.1	143.9	152.5	173	163.6	149.4	140.2	150.5	155.5	114.9	80.8	56.6	38.9	5.0	8.6
	1 STDEV Above Mean	508.9	520.8	539.0	568.4	555.2	520.3	465.5	459.9	415.2	353.2	226.0	117.6	79.2	15.9	21.6
Farm 3	4km Reflectivity	6	10	10	11	12	11	11	11	11	10	**9**	**9**	**9**	**9**	8
	Mean 4km Reflectivity	16.58	11.77	11.16	11.57	12.4	12.54	12.08	11.1	10.4	10.1	5.3	5.6	5.7	5.7	5.6
	Standard Deviation	14.47	7.606	6.375	6.659	6.594	6.957	6.965	6.8	6.8	6.0	2.9	2.8	2.8	3.1	3.0
	1 STDEV Above Mean	31.1	19.4	17.5	18.2	19.0	19.5	19.0	17.9	17.2	16.0	8.2	8.5	8.5	8.8	8.5
Farm 4	4km Reflectivity	22	12	14	13	13	13	13	5	4	5	6	6	7	5	5
	Mean 4km Reflectivity	16.58	11.77	11.16	11.57	12.4	12.54	12.08	11.1	10.4	10.1	5.3	5.6	5.7	5.7	5.6
	Standard Deviation	14.47	7.606	6.375	6.659	6.594	6.957	6.965	6.8	6.8	6.0	2.9	2.8	2.8	3.1	3.0
	1 STDEV Above Mean	31.1	19.4	17.5	18.2	19.0	19.5	19.0	17.9	17.2	16.0	8.2	8.5	8.5	8.8	8.5

DIAGNOSTIC INVESTIGATION OF A HIGH MORTALITY CASE IN COMMERCIAL ADULT TURKEYS IN THE CENTRAL VALLEY OF CALIFORNIA

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INTRODUCTION

High mortality events in commercial turkeys due to infection with one (1,2,3,4,5) or more virulent pathogens can have detrimental impacts on intensive poultry production (6,7). Introduction of virulent infectious diseases into poultry flocks can result in animal welfare and economic concerns associated with high morbidity and mortality (1,2,7). In addition, the added economic costs and labor associated with diagnostic surveillance, intervention strategies, and downtime can be significant (1,3,4,6). This report describes an event of acute high mortality in commercial, broad-breasted, white, 11-week-old meat turkeys due to co-infection with *Pasteurella multocida*, *Mycoplasma gallisepticum* and HEV, an immunosuppressive agent of turkeys.

MATERIALS AND METHODS

Four, 11-week-old commercial turkeys from one house were submitted to the California Animal Health and Food Safety (CAHFS) Turlock branch of the University of California–Davis, for postmortem examination and diagnostic work-up. Birds were submitted due to flock mortality increasing to 50% within one week. Antemortem findings included depression, ruffled feathers, and respiratory signs. The birds were humanely euthanized with CO2 gas and blood samples were collected to obtain sera for ELISA testing. Ancillary tests were performed according to standard operation procedures at CAHFS. Tissue samples were fixed in 10% buffered formalin for 72 h, embedded in paraffin, routinely processed and sections stained with hematoxylin and eosin. Microbial cultures from multiple organs were inoculated onto chocolate, 5% sheep blood and MacConkey agar plates, incubated aerobically at 7% CO2, and 37°C for 24-48 h. Intestinal scrapings were made to detect coccidia oocysts or parasite eggs. The oropharyngeal swab pool, tracheal swab pool and spleen tissue pool were tested by RT-PCR for avian influenza virus and by PCR for *M. gallisepticum* and *M. synoviae*, and HEV. Serum samples were routinely processed to detect antibody titers against *M. synoviae*, *M. gallisepticum*, Newcastle disease virus and HEV.

RESULTS

Grossly, there was hyperemia of the skin of the head, neck, and skeletal muscles, exudate in the respiratory system, severely enlarged, mottled livers, mildly enlarged kidneys, and severely enlarged, mottled spleens. Microscopically, there was extensive pneumonia with remarkable intralesional bacterial colonies (IBC) within affected parabronchi. Air sacs were expanded with exudate and scattered IBC. Epicardium showed extensive accumulate of exudate with scattered IBC. Spleens also showed extensive and severe lymphoid depletion.

No coccidial oocysts or parasite eggs were observed in intestinal mucosal scrapings. Pure cultures of *P. multocida* were isolated from livers and respiratory system. ELISA test revealed high antibody titers against HEV. HEV was detected in the spleen tissue pool from the submitted turkeys via PCR. *M. gallisepticum* DNA was detected from the tracheal swab pool and compared and differentiated from other *M. gallisepticum* field strains and vaccines by a multilocus-sequence analysis of the IGSR segment and *mgc2* gene. PCR results were negative for avian influenza and *M. synoviae*.

DISCUSSION AND CONCLUSION

The high susceptibility of commercial turkeys against both bacterial diseases and HEV diagnosed here are recognized. Moreover, the serologic response against HEV infection, could be linked to immunosuppression affecting these studied birds as lymphoid depletion was noted microscopically in the turkey spleen (8).

We reported a case of catastrophic mortality caused by co-*P. multocida*, *M. gallisepticum*, and HEV infections in commercial turkeys, with a mortality which was significantly higher than a previously reported case of highly pathogenic avian influenza outbreak in turkeys in 2016 in California (1). In addition to highly pathogenic disease agents, it is important to consider polymicrobial disease infections as potential causes of high economic losses.

ACKNOWLEDGEMENTS

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THE SOLUTION FOR BLACKHEAD IN TURKEYS IS THE PROBLEM

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OPTIMIZING INFECTIOUS LARYNGOTRACHEITIS VACCINATION VIA DRINKING WATER IN BROILER CHICKENS

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SUMMARY

Successful vaccination with infectious laryngotracheitis vaccines requires the virus to contact respiratory or conjunctival tissue. Vaccinating via drinking water in young broilers therefore has limitations in achieving successful initial uptake and generally requires some degree of spread of the vaccine virus through the flock. Studies in the field showed a high level of variation in the proportion of birds initially vaccinated and resulting problems with developing immunity. Studies using dye to show respiratory tissue contact indicated important practical techniques that affect the outcome of drinking water vaccination. The importance of rigorously following vaccination procedure recommendations was highlighted. The full-length article will be published subsequently.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a serious disease of the upper respiratory tract of chickens and is of particular concern when an outbreak begins in large populations of broiler chickens which are usually unvaccinated. The sheer numbers of birds involved requires mass vaccination techniques and this is commonly done via the drinking water route at 7-14 days of age. It is well known that an ILT virus, be it wild or vaccine strain, must contact upper respiratory tissue to cause an infection, and thus also to "vaccinate" the bird (1). The upper respiratory tissues include the conjunctiva, nasal mucosa, choanal cleft, eustachian slit, larynx and trachea. Contact with the mouth, buccal mucosa, tongue, pharynx, esophagus or crop and the gastrointestinal tract will not induce immunization. Hence by using drinking water it is hoped that some of the vaccine virus may be incidentally exposed to the upper respiratory tissues and is thus essentially "vaccination by accident" (1). Our field studies have indicated that ILT vaccine uptake by broilers vaccinated via drinking water is variable (2) and affected by administration technique (3). Experimental work to evaluate the importance of some vaccination procedures on vaccination "success" was therefore undertaken.

MATERIALS AND METHODS

Experiments were approved and supervised by the Animal Ethics Committee of the University of Sydney (ARA 2020/1834).

Application of drinking water vaccination is guided by the vaccine manufacturers' recommendations (4,5) and these are generally reflected in the broiler companies' Standard operating Procedures, with some minor variations between companies. Two experimental approaches were utilized. The first evaluated the amount of contact of drinking water with the upper respiratory tissues using vegetable dye to stain tissues contacted by drinking water under mock vaccination procedures. Variations of administration factors noted in field studies (3) were selected for study in a series of experiments. The variables studied included:

- Presence or absence of drip cups on drinkers
- Differing times of water deprivation (from 0 to 2 hours)
- Different times of activation of the flock by staff walking the house following vaccination (5, 15 or 40 minutes post mock vaccination)
- Different volumes of water / bird used for the vaccination process (15, 20 or 25 mL/bird)
- Repeat vaccination on a second day
- Increased water flow rate (54 mL/ m compared to 44 mL/ m)
- Lower height of drinkers to encourage splashing

Birds in small floor pens with nipple drinkers were mock vaccinated at 9 days of age with these procedures compared to a standard process which entailed: treated at day 9, 1-hour water deprivation, nipples with cups, 18cm nipple height from the litter, 30-40 mL/m flow rate, birds activated after 5 minutes. Three tissues were visually

assessed for staining by dyed water 1 hour after exposure: external nares, tongue and choanal cleft. The tongue is non-respiratory and only assessed whether the bird actually drank in the first hour; staining on the external nares demonstrates some water splashing but may not extrapolate towards much water entering the nasal passages. Therefore, only choanal cleft staining was considered indicative of successful contact with respiratory tissue for the purposes of these experiments. Several treatments were repeated on day 10. Red dye was used on day 9, blue on day 10 to overcome any confusion with carry over staining.

Based on the phase 1 results the second phase investigated the effect of different water deprivation times using actual vaccination with A20 ILT live attenuated vaccine (Zoetis) and testing choanal cleft swabs for the presence of ILT viral DNA by PCR on days 4 and 7 post-vaccination (pv). Therefore, in phase 2, birds were held in floor pens at typical industry stocking densities (24 birds per pen) and birds per drinker nipple (12 birds per nipple). From our field studies (3), 20 minutes was the average time of water deprivation used pre-vaccination. In this study, the Control pens had water withdrawn for only 20 minutes prior to vaccination at 9 days of age. The comparison treatment (termed the pre-deprived groups) had drinking water withdrawn for 1 hour at 8 days of age (24 hours prior to actual vaccination time) and then water was deprived for 20 minutes at 9 days of age prior to actual vaccine administration. In all other respects, the pens were subject to standard procedures: vaccinated at 9 days of age, drinker nipples with cups, 18cm nipple height from the litter, 30-40 mL/m flow rate, birds activated 5 minutes after vaccine exposure by staff walking near the pen. Plain water was supplied after 1 hour. Choanal swabs were collected following vaccination, replicated across three pens for each treatment on days 4 and 7 pv from both groups.

RESULTS AND DISCUSSION

In the dye studies the control groups (standard application procedures described above) showed no significant differences in choanal cleft staining (30.8%, 46.2% and 35.5%) across three separate experiments.

For choanal cleft staining following water sham vaccination on day 9, there were no significant differences between treatments and the control group on the same day except for lowered drinker height, where the lower drinkers had significantly less choanal staining (9.7% compared to 35.5% for the controls, P=0.015). Tongue staining and staining of the external nares appeared related. Not withdrawing water before administering dye gave the lowest percentage of birds with external nares and tongue staining and choanal staining was also numerically lower in this treatment than that of the control. There were no significant differences in choanal cleft staining with any other variations. Repeating the administration on day 10 resulted in tongue staining on the repeated day always being 100%. This indicated that birds were more likely to drink in the first hour on the second day of water deprivation. Staining of the choanal cleft was highly significantly increased (mean increase of 24.8% birds with staining) on the second day (Table 1).

One industry SOP suggests repeating vaccination over 2 days using half doses each time. Using a reduced dose rate is problematic as it has been long known that virus concentration presented to the respiratory tissues is important for the success of vaccination (6, 7). Vaccinating twice is unattractive to the farmer and requires a large time investment by service staff. However, we considered that it could be beneficial to simply withdraw water for 1-2 hours on the day preceding vaccination to encourage water access by the birds when the vaccine is administered.

In the phase 2 vaccination study, samples of the vaccinated drinking water taken from the distal nipple for each pen were assayed by qPCR for quantification of ILT vaccine virus presence and all were found to be similar. Results from PCR tests at day 4 pv evidenced one replicate with significantly higher virus detection in the control group, one replicate gave equivalent results, and the third replicate gave significantly higher detection in the pre-deprived group (Table 2). At day 7pv there were no differences between treatments. Overall detection averaged 50% of birds at day 4pv which was much higher than anything observed from the field studies. Hence the outcome was somewhat affected by the small-scale experiment being different from the field experience. To evaluate whether the proposed improvement possible from employing a water deprivation practice on the day preceding vaccination needs thorough evaluation in the field situation.

CONCLUSIONS

The studies highlighted the importance of achieving the manufacturers' administration recommendations. Specific findings instructed us of the importance of using water deprivation of at least 1-hour pre-administration and keeping drinker heights at the correct level. Evaluation of pre-depriving birds of water on the day before vaccination needs to be evaluated in the field.

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Table 1. Comparison of tissue staining when mock vaccination was repeated the following day

	% birds with stained tissue when sham vaccinated on day 9			% birds with stained tissues when re-vaccinated on day 10 for the same treatment		
Treatment	External nares	Tongue	Choanal cleft	External nares	Tongue	Choanal cleft
No cups	50.00	64.71	20.59	91.18	100.00	55.88
Cups (control)	73.08	76.92	30.77	84.00	100.00	72.00
5 min. activated	86.67	90.00	30.00	100.00	100.00	39.29
15 min. activated	92.59	92.59	18.52	74.07	100.00	51.85
40 min. activated	78.57	75.00	32.14	74.07	100.00	37.04

Table 2. Comparison of percent positive choanal swabs from Pre-deprived and control pens following A20 ILT vaccination.

		% choanal cleft swabs positive for ILTV			
Room	Treatment	Day 4 pv		Day 7 pv	
2	Predeprived	30.4	B	34.8	
	Control	62.5	A	54.5	
4	Predeprived	41.7		45.8	
	Control	37.5		54.2	
8	Predeprived	87.5	A	45.8	
	Control	45.8	B	59.6	
A,B: means within the same room with different postscripts differ (P<0.05, Fisher's exact test, 2-tailed)					

CROSS PROTECTION WITH A LIVE ATTENUATED *SALMONELLA* TYPHIMURIUM VACCINE TO PROTECT AGAINST A PREVIOUSLY UNIDENTIFIED STRAIN OF *SALMONELLA* ENTERITIDIS

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SUMMARY

Salmonella serotypes vary throughout the livestock industry. Historically *Salmonella* Enteritidis is not considered to be prevalent in Australian poultry flocks. As recently as 2018 a novel S. Enteritidis emerged in the industry (1). There are only unregistered autogenous bacterins vaccines available in Australia against this serotype in poultry. Could the use of a registered live attenuated Salmonella vaccine have the potential to cross protect against several Salmonella serotypes be a solution for the industry?

INTRODUCTION

Currently, if a commercial layer flock becomes infected with serotype Enteritidis, flock depopulation is mandated, and the farm must demonstrate freedom before restocking. This has a significant animal welfare and economic impact on farms where this serotype has spread, representing a significant risk to the Australian poultry industry and human health. The benefits of an existing live attenuated *S.* Typhimurium vaccine (Vaxsafe ST®) to be able to protect birds against both *S.* Typhimurium and *S.* Enteritidis challenge in the field would increase confidence and security in the industry and enhance human protection against food borne salmonellosis.

MATERIALS AND METHODS

One hundred sixteen commercial layer hens were held in floor pens much like commercial production practices. The birds were either vaccinated using a live *Aro*-A deletion mutant vaccine following the manufacturer's recommended program or this program with an added intramuscular administration or left unvaccinated (control) against *Salmonella*. Commercial layers were assessed at point of lay at 18 weeks of age, after sexual maturity, as this is a high stress period where the layers are assumed to be most vulnerable. The hens were challenged with the novel *S.* Enteritidis strain (phage type 12 according to the *Salmonella* reference laboratory IMVS) orally at 10^9 colony forming units/bird.

To establish a cross-protection benefit, the vaccination program applied was as follows: day old (coarse spray), three weeks age (oral gavage), followed by either one or two intramuscular doses of the same vaccine at nine, or nine and 14 weeks of age respectively. Protection was measured by a reduction in the proportion of *S.* Enteritidi*s* detected in cloacal swabs, ceca, liver, spleen, and reproductive tracts of unvaccinated and vaccinated birds. Antibody titer test were conducted for Group B and Group D serovars to measure the effect of vaccination on the production of these antibodies. The results shared here will focus on results from cloacal swabs.

RESULTS

Results from cloacal swabs are: in the challenged groups, all had a similar proportion of cloacal swabs positive at three days post challenge (83%), but this declined significantly by days 10 and 14 post challenge. The proportion of birds with positive cloacal swabs at 5 days post challenge was significantly lower in the single-injection vaccinated group and at seven days post challenge in the double-injection vaccinated group compared with the challenged controls.

DISCUSSION

This research assessed the efficacy of the vaccine in reducing systemic infection and fecal excretion of *S.* Enteritidis organisms in challenged layer hens. The vaccinations are considered effective if there was a significant reduction in the number of cloacal swabs from vaccinates containing challenge organisms compared to the number from the controls. This can be noted in Table 1, where 5 day cloacal swabs from single vaccinated birds and 7 day cloacal swabs from double vaccinated birds show significance against unvaccinated challenged birds.

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Table 1. Cloacal swab results for 116 commercial layers; The number of cloacal samples positive for *Salmonella* Enteritidis. Control group (unvaccinated and unchallenged) remained Salmonella free throughout the trial. At 18 weeks of age all three groups were challenged orally with the novel *S.* Enteritidis strain (109 cfu). Vaccinated Regime 1 depicts a single intramuscular vaccination and Vaccinated Regime 2 depicts a double intramuscular vaccination.

Treatments	No. Positive Results Day 3,5 & 7 (n=32) Day 10 & 14 (n=16)				
Day 3,5 & 7 N= 32 per treatment Day 10 & 14 N=16 per treatment	3 Day Cloacal Swab	5 Day Cloacal Swab	7 Day Cloacal Swab	10 Day Cloacal Swab	14 Day Cloacal Samples
Unvaccinated	27	26	21	8	2
Vaccinated (Regime 1)	28	18*	18	6	3
Vaccinated (Regime 2)	25	22	12*	7	2

*Significantly different to unvaccinated challenge group (<.05)
(P<0.05, Fisher's exact test, 2-tailed)

NEW INSIGHTS FOR TOTAL CLEAN-OUTS – WHAT WE CAN LEARN FROM WELL-MANAGED SYSTEMS

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SUMMARY

Litter maintenance and management is critical for long-term use. However, either through individual farm issues or chronic bacterial challenges, difficult litter management decisions need to be considered like total clean-out or expensive litter amendments. Total clean-outs offer a system to remove, sanitize, and re-start environmental systems. To this end, the impact of resident bacterial populations is dramatically altered. Bacterial communities form in a consecutive process that can ultimately result in a defensive position of the total community towards exogenous bacteria. Windrowing or other between-flock management strategies shift bacterial populations, but still resolve key populations that re-establish quickly and maintain more homeostatic relationships. Total clean-out, and even more severe sanitization practices, result in bacterial competition to re-establish in the environment – dependent on loading, inputs, and chemical resistance. Here we present a proof-of-concept study to evaluate bacterial changes in management practices for litter. Trends in data indicate potential for pathogens to establish within the general population and trends in bacterial systems throughout the life of the flock. A multi-flock analysis continued to demonstrate similar findings in data in that bacterial populations require time to establish themselves, while also supporting unique critical control points. The application of this data may be important in evaluating litter management practices, decisions around re-seeding litter environments, and between-flock actions.

INTRODUCTION

Litter is a medium that allows for significant growth of microorganisms and sustained density. The source of microbial growth in litter is related to a consistent source of deposited bacteria and carbon/nitrogen sources that secure metabolism across the microbiome. Water is another factor that is important to microbial growth in litter and has a strong relationship to limiting input for bacteria to replicate or maintain density (1).

Bacteria, as a microbiome, naturally cycle in the litter environment all regulated through both the microenvironment and available nutrition (2). Also affecting bacterial populations can be significant and sudden changes in physical nature of the litter like composting, temperature changes, and reduced input from poultry manure. Downtime and management changes when birds are absent help to account for potential microbiome swings.

Litter management in integrated poultry systems usually demonstrates a distribution of management practices – both instructed from complex leadership and individual growers. The distribution of practices leads to several interactions with the microbiome. Those interactions reveal either a natural reduction in density and bacterial populations or can lead to increased bacterial density (3,4). The microbiome environment can be a significant predictor for interactions in poultry performance and health. This relationship in health is due to chicks placed in litter environments and their sensitivity to their forming microbiome (5).

Predictability is much sought after with numerous challenges that can arise with production. Although much effort is placed on raw data for statistical conversion, predictability can be much simpler with understanding of risk and impact of weighted variables. Litter management is one such variable that could be used with high weight for production – indicating that predictability is inherent with litter condition (6). Before technology is deployed, litter and environmental management can give an indication of influence on success. To this end, health technology deployed on a single-farm basis can have a direct relationship to value regarding the management status of the farm.

The work presented here is in response to exploring predictability of farm management practices with established biomarkers of microbial populations. *Escherichia coli* is a hallmark of microbial populations within poultry production and is related to both environmental and gut-associated factors (7). Studying *E. coli* allows for a single metric across factors of management to explore how changes can be interpreted as an effect on bacterial maintenance. Of particular importance is the relationship on total clean-outs of the poultry environment compared to in-house composting. Furthermore, in-house composting methods may also have specific differences in their outcomes on key bacteria. A

series of commercial studies is presented here to help explain documented differences in disease risk based on common clean-out or compositing practices.

MATERIALS AND METHODS

Trial and sampling design. Farms sampled in the program were maintained within a single integrator complex system to standardize genetics, feed, and cross-complex management. The farms used in this study are located in the southern region of the United States. Study designs included designation of methodology and routine sampling throughout the grow-out process. For study 1, a single farm (2 farms assigned to each treatment) was chosen to investigate total clean-out of litter from houses compared to windrowing in houses. Those houses that received total clean-out was only performed before flock 1 and then managed through basic litter upkeep procedures between flocks (Cake-out). Windrowing houses performed this method between each flock placement. For study 2, again a single farm was chosen to be monitored over five separate flocks. Methods for downtime litter conditioning were performed between each flock placement. Pre-flock placement sampling includes samples 24hours before chick placement, while flock sale samples includes samples within 48h of houses after catch.

Bacterial quantification from litter samples. Litter samples were added to 100mL flask of 10% TSB and shaken for 1h at room temperature. The sample were filtered to remove solids. 100ul samples were inoculated onto spiral plated agar plates of TSB, CHROMagar, and BHI across a dilution scheme. Conditions for incubating samples included 21% oxygen to 0% oxygen exposure and utilized 37ºC. Plate counts of bacteria were measured using photometric software.

qPCR of bacterial virulence genes. From individual isolates *E. coli* and *C. perfringens*, which is deposited into a Zymo Research DNA/RNA Shield Collection Tube (R1107) with 1mL fill. The samples were processed according to manufacturers instructions to yield purified gDNA. The gDNA originating from avian microbiome samples is tested against 14 targets, including 11 *E. coli* (EC) genes (iss, tsh, iucC, cvaC, irp2, iutA, traT, ompT, ibeA, irp2, uidA), two *Clostridium perfringens* (CP) genes (CPA and netB), and another marker, 16S T1. uidA is a general EC marker which is used as a proxy for enumerating EC on the swab. CPA is a general CP marker which is used for the same purpose. Similarly, 16S T1 is a general bacterial marker, used to enumerate total bacteria.

RESULTS

Bacterial growth is regulated by nutrition, physical space, and interactions from other colonizing microorganisms. Such growth media as chicken litter readily supplies bacteria with an abundance of nutrient sources, therefore, secondary relationships of pre-existing bacterial populations or physical space start to have significant relevance to bacteria growth. Methods of litter conditioning like windrowing or decaking create physical alterations in the litter that can create small changes in the bacteria that make up the population (Fig 1.). However, with existing bacterial relationships to initiate reactions after the microenvironment stops changing - the bacteria are able to quickly equilibrate. Total-clean out events naturally remove the self-regulation of bacterial populations, while leaving abundant nutrients. Log-density changes in *E. coli* shows relationships are not readily established when the bacterial relationships are shifted quickly.

To assess the dynamics of *E. coli* from downtime through harvest via management practices, a farm was studied over three separate flocks for the bacterial density in their litter. Sampling was accomplished by harvesting litter over segmented quartiles over the length of the poultry house. From Flock 1, one house completed at total clean-out while a paired house completed windrowing as individual downtime practices. In Flock 2 – 3, total clean out received basic decake methodology while the other paired house continue to perform windrowing between flocks. As a time-course, *E. coli* showed typical bacterial growth from very low density from downtime up to a steady-state at day 7 (Fig 2.) Total clean-out house did not demonstrate steady-state in densities as compared to the windrow house. Using average densities among all three flocks, the densities of *E. coli* showed log changes among total clean-outs compared to the windrow house.

Of the in-house composting methods, cake-out and windrowing both offer mechanisms of aerating litter and supporting reduced litter moisture. Compositing is also key to reducing key bacterial populations between flocks. However, both of these methods are used and predicated on regional, seasonal, disease issues. To investigate ranking the benefits of these methods, a 5-flock series was measured by standardizing these actions across the same farms (Fig 3.). Pre-flock placements as a measure of acute changes were used in a time series while also including measurement of litter at the time of sale. *E. coli*, avian pathogenic *E. coli* – designated with 2 or more virulence genes from panel, and Clostridium perfringens were calculated to give insight into commensal and pathogenic bacteria. Although some time-points within the 5-flocks showed differences between either specific bacterial group or collective trend, gross

changes from multi-log differences were not identified. Trends of pre-placement bacterial density to density at sale of flock as a log difference did show similar levels of bacteria, but were different between flocks. First and final flocks showed close bacterial density regardless of composting method.

Studying *E. coli* helps to demonstrate general effects against bacteria that tolerate oxygen in the environment. *C. perfringens* is an obligate anaerobe and requires protection from oxygen in the environment. The biology of *C. perfringens* also includes sporulation and vegetative forms, which can cycle depending on challenges in the system. *C. perfringens* is not estimated to be remediated from a system because of its ability to sporulate. Therefore, its cycling can help to monitor significant challenges to its growth. Both windrowing and cake-out had an impact on *C. perfringens* remaining withing the production environment, but both methods caused different events between flocks (Fig 4.). The largest differences between samples appear to be from Flock 1 and Flock 2, while Flock 5 also showed separation. It is unclear whether availability of oxygen, physical changes in the litter, or microbial population shifts are related in these shifts in *C. perfringens*.

Downtime is essential to organize parameters before animals are placed in the system. Consistency in certain downtime metrics like reduced bacterial populations or certain litter physical/chemical measurements help to reduce risk. Of the 5-flocks that were supervised, consistency in downtime was measured between *E. coli*, APEC, and *C. perfringens* densities in litter (Table 1). An interesting finding of the limited effects of downtime litter conditioning was present between the bacterial isolates. However, further management between flocks showed a consistent effect in key bacterial reduction regardless of program. Trends between the conditioning methods do not show appreciable differences on bacterial densities during downtime of the flocks.

Relationships between *E. coli* and pathogenic forms of the bacteria require acquisition of virulence genes that support disease activity. However, these virulence genes have relationship to fitness in the system. If virulence and disease activities supports their function in a host or environment, then the genes will be maintained. When fitness does not support holding of virulence genes, the genes are often reduced from the population. To study virulence gene trends in *E. coli* compared to methods of litter condition, isolates were measured for their density of target virulence genes – denoting pathogenic isolates as those that have >2 virulence genes of ten that are assayed (Fig 5.). Cake-out and windrow methods both showed similar ratios of virulence genes in *E. coli* from early and later flocks.

DISCUSSION

Highlighted by the effects of total clean-out of litter compared to windrowing as a litter conditioning method, an established bacterial community that is allowed to maintain maturity showed greater ability to self-regulate bacterial densities. Mature bacterial communities are interdependent on structure of metabolites, microenvironment, and other interactions between bacteria in the same space. What is becoming more understood is that this maturity yields a community phenotype of self-protection. This self-protection may involve regulating nutrition or space at which introduced bacteria can interact with the community. Bacteria in the poultry production environment form these communities and move to maturation. These bacterial relationships can be maintained with the consortia feeding into community establishment. However, total clean-outs remove these relationships between the bacteria. Once removed, bacteria must start the process of self-organization over again resulting in usual competition for resources and space. Total clean-outs have some utility in removing severe pathogen build-up or potential mis-regulation of the Eimeria cycling process to fuel immune priming. Although given this potential need to reduce pathogens, windrowing between flocks to condition litter demonstrates the ability to preserve bacteria in the litter and regulate a typical bacteria – *E. coli* – throughout a flock. After total clean-out, relative densities of *E. coli* compared to windrowing were re-established after three consecutive flocks performing in-house litter conditioning.

With evidence of litter conditioning between flocks showing a positive effect on bacterial communities, variations of litter conditioning was investigated. Windrowing, as investigated from trial set one, can maintain bacteria from flock to flock. Cake-out is another mechanism that involves aeration and changes to the physical nature of litter without specific antagonist measures against the bacteria. Between litter conditioning methods, no gross changes in bacterial density were identified – using both biomarkers of *E. coli* and *C. perfringens* density. Total *E. coli* and pathogenic *E. coli* also showed similar changes between litter conditioning methods on a long-term flock study, with initial changes found after flock two. Just from basic bacterial studies, the utility of either method did not show significant differences that would have the potential to shift decision-making within commercial production.

One specific hypothesis around application of windrowing compared to cake-out may be related to consistency of the management. Large-scale management of farms across a complex requires the intersection of complex management and individual grower management. It is widely understood that management across a complex is displayed as a distribution for those that are intensely managed and those that are poorly managed. The performance of this trial required specific supervision between flocks with litter conditioning performed under close inspection.

This may not be the true implementation of techniques under commercial conditions, but it does yield data to represent when the methods are performed well and consistently.

Virulence of the biomarker strains help as a methodology to study fitness of bacteria. As bacteria acquire virulence genes, the acquisition comes at a cost of fitness unless selection pressure supports use of the virulence genes. The preservation of virulence genes in a system can show if infection pressure or constant introduction of pathogenic *E. coli* is being supported. Bacterial communities, fitness over resources, or increased bacterial density can change the ability for *E. coli* to maintain these virulence genes. Windrowing or cake-out did not overall change pathogenic *E. coli* within the litter system during grow-out of a flock. A hypothesis going into these studies included litter conditioning supporting reduced pathogenic bacteria. However, it does not appear that this type of intervention alone can shift pathogenic bacteria. For future applications, litter conditioning could be used with other litter amendments to have an improved effect on limited bacterial pathogens.

ACKNOWLEDGEMENTS

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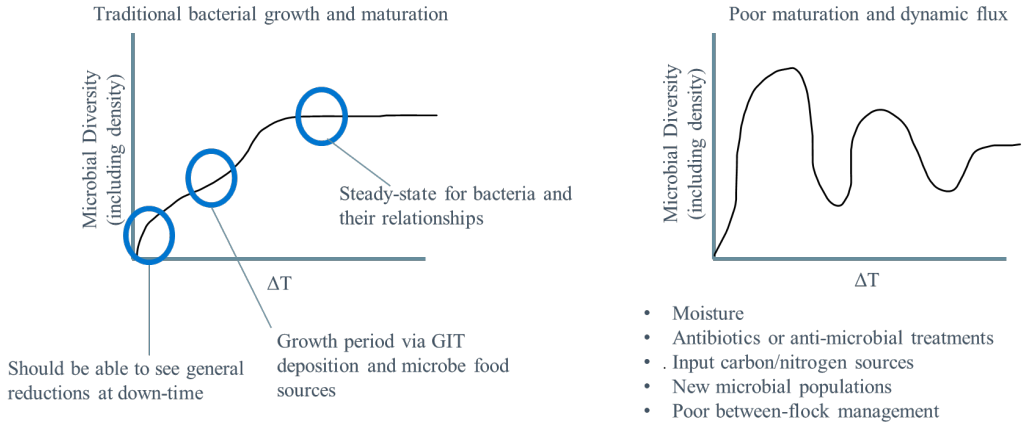

Figure 1. Models of microbial populations within steady-state and dynamic changes in the poultry environment. Bacterial growth is predicated on growth factors and traditional growth parameters. Steady-state growth dictates predictive characteristics of growth and kinetics to a point at which both growth and density are regulated by populations and metabolites. Dynamic change results in severe changes in bacterial density versus available growth factors that otherwise result in high growth or reduced growth over time.

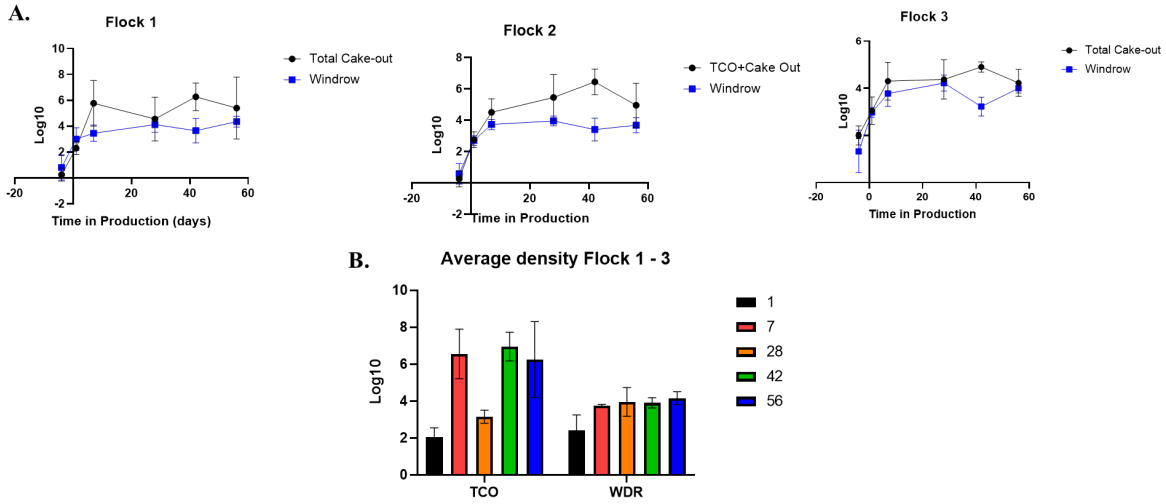

Figure 2. Total clean-outs compared to windrowing downtime management practices on E.coli litter densities. E. coli density measured at pre-placement (-4 days) up through harvest time (56 days) through 3 consecutive flocks on the same farm (A). Averages measured from quartile sampling with the house. Total density averages were compared over each time point between management practice (B).

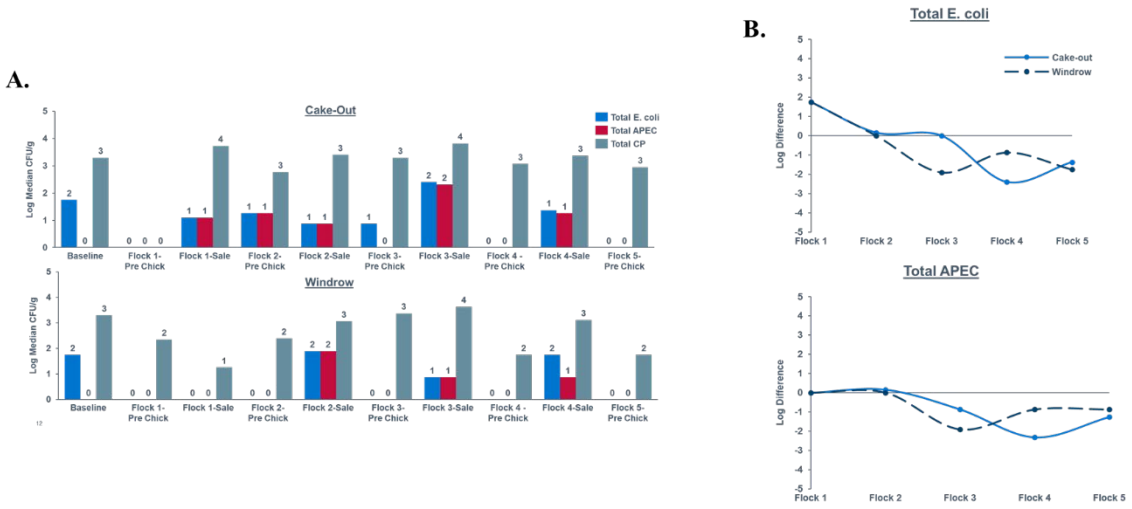

Figure 3. In-house composting methods of cake-out or windrowing as compared through E. coli recoveries. 5-flock summary of E. coli and C. perfringens from litter of pre-flock placement and sale (A). Log medians listed from recoveries. Trends between flocks of pre-flock placement and sale as log difference between recoveries (B).

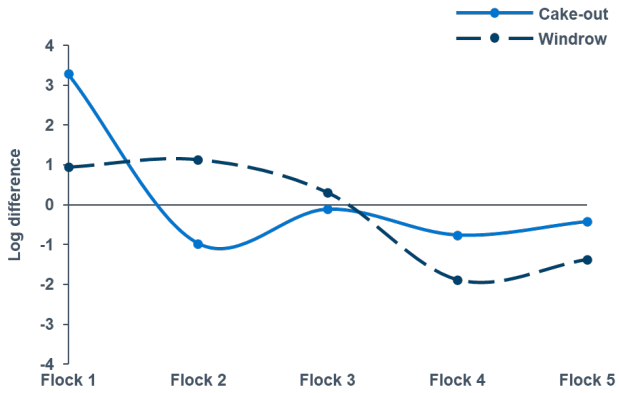

Figure 4. In-house composting methods and recover of C. perfringens in successive flocks. Density of C. perfringens measured between pre-flock placement and at harvest. The quantitative difference between pre-placement and harvest shown as log difference. Methods in composting were maintained and performed before each pre-flock placement.

A.

	TRT	Δ log E.Coli	Δ log APEC	Δ log Clostridia
Flock 1	Cake-out	+1.75	0	+3.29
Flock 2	Cake-out	+0.16	+0.16	-0.97
Flock 3	Cake-out	0	-0.87	-0.11
Flock 4	Cake-out	-2.40	-2.32	-0.76
Flock 5	Cake-out	-1.37	-1.26	-0.42

B.

	TRT	Δ log E.Coli	Δ log APEC	Δ log Clostridia
Flock 1	WR	+1.75	0	+0.95
Flock 2	WR	0	0	+1.14
Flock 3	WR	-1.90	-1.90	+0.31
Flock 4	WR	-0.87	-0.87	-1.88
Flock 5	WR	-1.75	-0.87	-1.37

Table 1. Between-flock measurements of bacteria based on in-house conditioning methods. Bacterial density measured during down-time of flocks after conditioning method applied. The differences listed are from prior flock to current measured flock. Negative changes are listed as growth of bacteria from prior flock, while positive changes listed as reduced bacterial growth. No real changes in bacterial growth listed as net neutral.

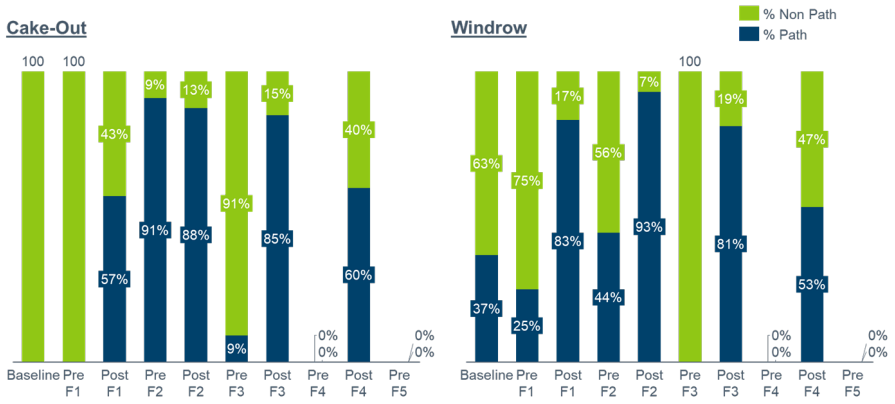

Figure 5. Commensal to pathogenic bacteria ratios between litter condition methods. Pre-flock placement and harvest of measurements of commensal E. coli and pathogenic E. coli. Pathogenic E. coli denoted by qPCR of target pathogen genes - >2/10 pathogen genes was identified at pathogenic. Total density was set to 100% total E. coli population per sampling point.

INVESTIGATION OF A SPOTTY LIVER DISEASE OUTBREAK IN BROILER BREEDERS IN SOUTHEAST USA

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SUMMARY

In the first quarter of 2022, several broiler breeder flocks raised in a commercial operation in Southeast United States experienced elevated mortality and decreased egg production at the onset of lay. Necropsy of dead hens revealed multifocal 1-2mm, white-grey-cream-colored lesions in the liver, consistent with Spotty Liver Disease (SLD). Bile and liver samples were collected for bacteriological analysis. *Campylobacter hepaticus* was isolated from bile samples and further characterized by phenotyping and whole genome sequencing using Illumina MiniSeq. Antibiotic sensitivity test of these isolates was performed. Reduction in hen mortality was observed after receiving antibiotic treatment and no indications of SLD were noticed through Summer 2022. In the last quarter of 2022, Spotty Liver Disease re-occurred in the first affected farm. Case details and results of the epidemiological investigation are presented in this report. To the authors' knowledge, this is the first documented report of Spotty Liver Disease in US broiler breeders.

INTRODUCTION

Spotty Liver Disease is an acute infectious disease of layer chickens characterized by multifocal necrotic lesions in the liver, increased mortality, and drops in egg production (1). Although the disease has been recognized since the 1950's (known as Avian Vibrionic Hepatitis), its etiological agent *Campylobacter hepaticus* was not identified till recently (1, 2). The disease is becoming more prevalent in Europe and Australia, especially in cage-free layers. SLD caused by C. hepaticus was first reported in layer chickens in the United States by Gregory et al. in 2018 (3). Since then, *C. hepaticus* has been isolated from many table egg layer flocks in the USA with clinical SLD (Ceva SSIU Database). Although SLD has been mentioned to affect meat breeder flocks in the literature (4), details of these cases are scarce. The objective of this paper is to describe a recent outbreak of Spotty Liver Disease in Broiler Breeders in Southeast USA.

MATERIALS AND METHODS

Case history. A total of 31,264 Cobb 500 pullets and 4,168 Hubbard M-99 cockerels raised in Farm 1 were moved to two different breeder farms (Farm A and Farm B) on October 25, 2021, at 21 weeks of age. In Farm A, weekly hen mortality increased to >1.2%/week at 26-28 weeks of age and peak egg production only reached 75%. These issues were not observed in Farm B. Post-mortem examination of dead birds in Farm A revealed multiple small pale lesions in the liver consistent with SLD. On November 24, 2021, four hundred thirty 25-week-old males from Farm A were brought to Farm C and introduced as spike males in 3 houses of 51-week-old broiler breeders. Two weeks after spiking those flocks, egg production dropped by 10-15%, similar liver lesions were observed but hen mortality did not increase. *Campylobacter hepaticus* was not detected in these birds.

On January 3, 2022, 33,163 Cobb 500 pullets and 3,543 Hubbard M-99 males raised in Farm 2 were moved to two different breeder farms (Farm D - 2 houses, and Farm E - 1 house) at 21 weeks of age. As described in Farm A, weekly hen mortality increased to >1.2% by 26-29 weeks of age, and peak production was ≤70% in all 3 houses. Dead and sick hens were examined at 29 weeks of age. Multiple, small, cream-colored lesions in the liver were observed (Chart 1). Bile and liver samples were taken from these birds and submitted for bacteriological analysis to the Scientific Support and Investigation Unit (SSIU) at Ceva Animal Health, Lenexa, KS. Affected liver tissues were also collected for histopathological examination.

All flocks mentioned above responded positively to antibiotic treatment via drinking water (neomycin and oxytetracycline, 10mg/lb of body weight) administered for 5 days after clinical signs were noticed. On February 25,

2022, chlortetracycline was introduced in the breeder feeds at 400g/ton and administered every other week across the operation until October 31, 2022.

On September 19, 2022, another 21-week-old flock raised in Farm 1 was moved again to Farm A (22,228 Cobb 500 pullets and 2,320 Cobb 500 cockerels), and Farm F (11,008 Cobb 500 pullets, and 1,123 Cobb 500 cockerels). These farms never received breeder feed medicated with Chlortetracycline. While hens in Farm F did not experience any increased mortality or egg production drops, hens at Farm A were behind on egg production (<73%) at peak and showed liver lesions consistent with SLD (Chart 2) at 28 weeks of age. Liver and bile samples were collected from affected hens and submitted for bacteriological analysis. Due to early recognition of the problem, antibiotic treatment (neomycin and oxytetracycline, 10mg/lb of body weight) was administered to these flocks for 5 days via drinking water and they responded positively.

Bacterial culture. Isolation of *C. hepaticus* was performed with modifications of methods by Crawshaw and Van (1, 2). Briefly, 1 mL of bile fluid or 1 g of aseptically macerated liver was added to 9 mL of modified Preston Broth. Cultures were incubated at 39-41°C for up to 14 days in 8-10% CO2 incubator. Preston Broth cultures were subcultured to Brucella Agar plates containing 5% horse blood (BHB) (Thermofisher, Waltham, MA) periodically from 3-14 days post inoculation. Inoculated BHB plates were incubated similarly for up to 14 days. Microaerophilic bacterial culture was performed on Tryptic Soy with 5% Sheep Blood Agar plates (Hardy Diagnostic), Chocolate Agar plates (Hardy Diagnostic) and MacConkey Agar plates (Hardy Diagnostic) followed by streaking for isolation and incubated in 5% CO2 at 33-37°C for up to 48 hours. Isolated strains were frozen for storage in tryptic soy broth (Sigma-Aldrich, St. Louis, MO) with 25% glycerol. Antibiotic sensitivity test was performed at the Veterinary Diagnostic Laboratory of Iowa State University.

Phenotyping bacterial isolates. Suspected *C. hepaticus* strains were subcultured on Brucella Agar plates at 39-41°C for 2-3 days until sufficient sized colonies could be tested using API® Campy identification system (bioMerieux, France).

Whole genome sequencing of *Campylobacter hepaticus*. Genomic DNA was extracted from suspect isolates using MagMAX™ CORE AgGenomic DNA Extraction kit (ThermoFisher). Whole genome sequencing was performed using Illumina MiniSeq. Raw sequences were mapped to the 16s rRNA gene of *C. hepaticus* HV10 reference strain CP031611 (GenBank accession no. KU886019.1) using Geneious Prime® version 2022.1.1. The resulting contigs were identified using 16s ribosomal RNA BLAST search at the National Center for Biotechnical Information (NCBI). Raw sequence data for each strain of the present case, and strains from our collection, were normalized and error corrected prior to assembly using SPAdes version 3.15.3. Denovo assemblies were assessed for epidemiological relatedness by single nucleotide polymorphism (SNP) analysis using SNIPPY version 4.6.0. Core SNP alignment files were visualized using Geneious Prime. Virulence genes were analyzed using ABRICATE to search the virulence factor database (VFDB) (4).

RESULTS

Histopathology. Liver samples from affected hens in Farm D and E had acute multifocal necrotizing hepatitis characterized by disseminated discrete foci of fibrinoid necrosis of hepatocytes, variably attended by heterophils and small mononuclear inflammatory cells (Chart 3). The sinusoids were diffusely congested. These findings in liver were morphologically consistent with hepatitis caused by *Campylobacter hepaticus* (1).

Bacterial culture and whole genome sequencing. A summary of *Campylobacter hepaticus* culture and whole genome sequencing results is shown in Table 1. *C. hepaticus* was isolated from bile samples from hens in Farms D, E, and A, and was detected by qPCR in liver from hens in Farm A. Whole genome sequencing indicated that *C. hepaticus* isolates from Farms D, E, and A were highly similar to the *C. hepaticus* HV10 reference strain. Other bacteria found in livers from hens in Farm A were *Escherichia coli*, *Enterococcus cecorum*, and *Gallibacterium anatis* biovar hemolytica.

Antibiotic sensitivity test. *Campylobacter hepaticus* isolates from Farm D and E were sensitive to enrofloxacin, florfenicol, gentamycin, neomycin, sulphadimethoxine, streptomycin, and sulphathiazole. *C. hepaticus* from Farm D was resistant to trimethoprim/sulphamethoxazole, but sensitive to ceftiofur and penicillin, while opposite results were found for *C. hepaticus* isolate from Farm E. No guidelines have been established by the Clinical and Laboratory Standards Institute (CLSI) for the interpretation of *C. hepaticus* combination with certain drugs (i.e. amoxicillin, clindamycin, erythromicin, novobiocin, oxytetracycline, spectinomycin, tetracycline, and tylosin). Field evidence suggests that treatment with neomycin/oxytetracycline via drinking water, or chlortetracycline in the feed were effective controlling SLD in Farms C, D, E, and A.

DISCUSSION/CONCLUSION

In this case report, SLD associated with *Campylobacter hepaticus* has been demonstrated in broiler breeders. As previously reported by other authors (5, 6), the disease occurred at the onset of lay. SLD has been commonly reported during hot weather (4), but that was not the case for these outbreaks, coinciding with recent epidemiological observations from Phung et al. (5) in which the disease occurred all year round.

Whether the pullets were infected with *C. hepaticus* before being moved or got infected in the hen house is unknown in these outbreaks. Existing research (6) has shown pullets infected with *C. hepaticus* during rearing as early as 12 weeks of age, and two weeks before overt clinical signs of SLD were noticed in a laying flock. It has been hypothesized that predisposing factors like physiological or gastrointestinal microbiota changes associated with the onset of egg production may contribute to the occurrence of SLD in asymptomatic birds previously infected with *C. hepaticus* (6). This could be the case for the SLD outbreaks at Farms D and E described in this report, receiving pullets from the same farm (Farm 2).

C. hepaticus has also been detected in environmental samples (dust, soil, water) and vectors (flies, wild birds, rodents, mites) (6) so potential contamination from those sources should also be considered, especially in farms where SLD has occurred in subsequent flocks like Farm A in this report.

Since SLD has been often described in table egg layers where there are no males in production, and there is not much evidence of the disease affecting broiler breeder males, the role of spike males in Farm C outbreak is unknown. However, the potential risk of spike males transmitting SLD to broiler breeder hens in lay should not be overlooked.
Although antibiotic treatment has been an effective tool to control SLD in the affected broiler breeder flocks, biosecurity measures have been enhanced to prevent the spread of this bacteria (extended out time, cleaning and disinfection, water line sanitation, dedicated footwear). Based on the SLD occurrence pattern at this operation, monitoring the presence of *C. hepaticus* in pullets and hens before lay at farms where SLD has already occurred is planned to better understand this disease in broiler breeders.

From a clinical standpoint, and based on the findings presented above, Spotty Liver Disease due to *C. hepaticus* should be considered when broiler breeder hens experience increased mortality, decreased egg production, and multifocal liver lesions at the onset of egg production.

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Table 1. *Campylobacter hepaticus* culture and whole genome sequence results from bile and liver samples form SLD-affected broiler breeder hens.

Farm	Hen Age (weeks)	Sampling Date	C. hepaticus isolation	C. hepaticus qPCR	Virulence Factor Database match to C. hepaticus HV10 Reference strain
D	29	March 3, 2022	+ Bile	+ Bile	100% identical (69/69 virulence factors)
E	29	March 3, 2022	+ Bile	+ Bile	100% identical (69/69 virulence factors)
A	28	November 9, 2022	+ Bile	+ Bile & Liver	100% identical (69/69 virulence factors)

Chart 1. Multifocal, small, cream-colored lesions in the liver of a 29-week-old broiler breeder hen with Spotty Liver Disease from Farm D.

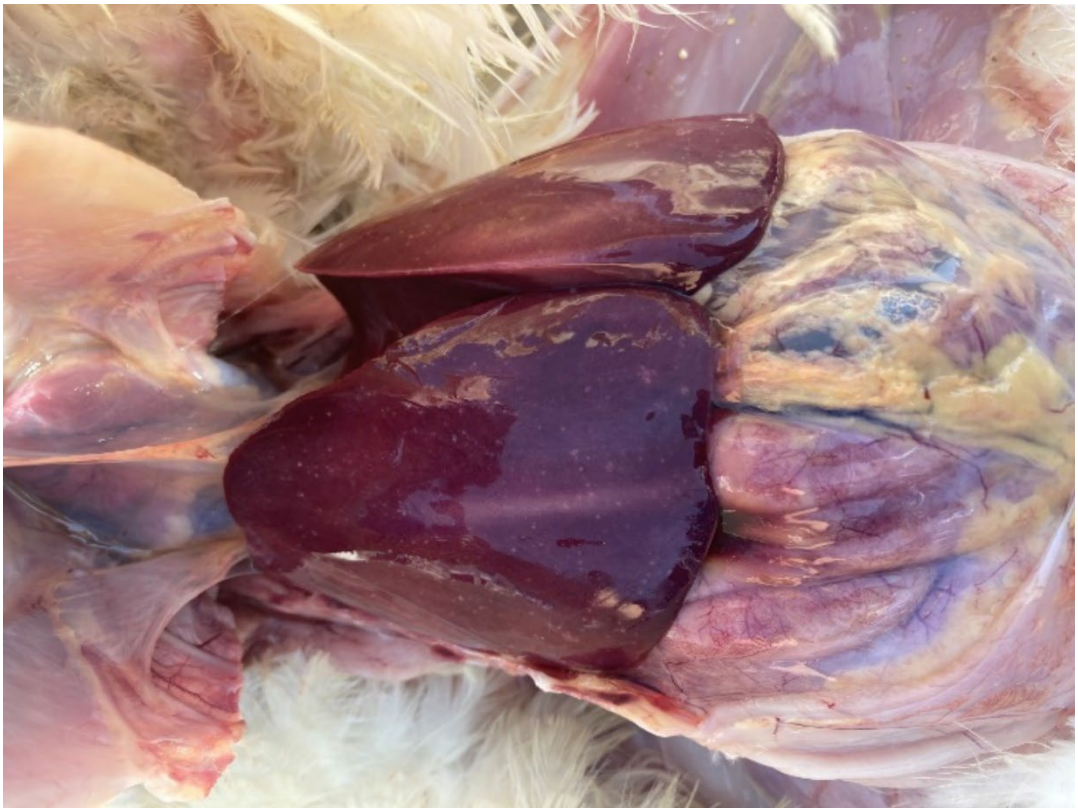
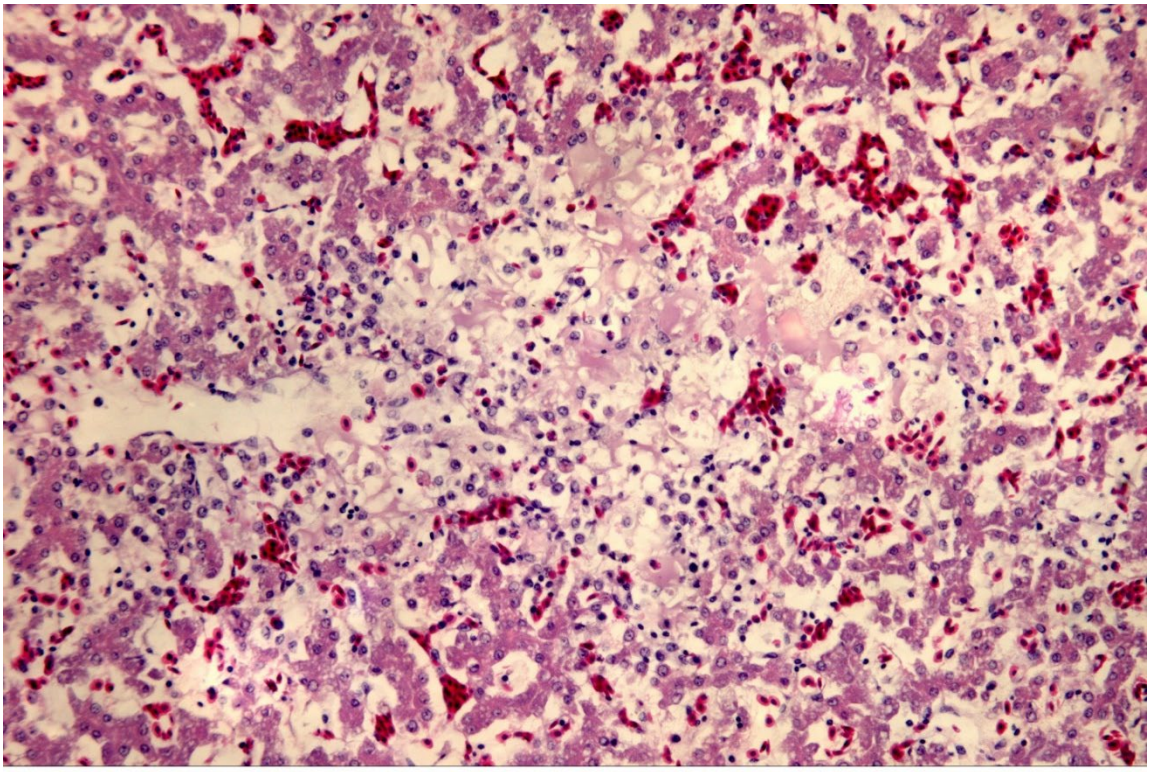


Chart 2. Multifocal, small, cream-colored lesions in the liver of a 28-week-old broiler breeder hen with Spotty Liver Disease from Farm A.

Chart 3. Acute multifocal necrotizing hepatitis in the liver of a 29-week-old broiler breeder hen with Spotty Liver Disease.



THE CLINICAL APPLICATION OF NANOPORE SEQUENCING TO CHARACTERIZE A NEW DMV/1639-LIKE STRAIN OF IBV DETECTED IN IOWA

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SUMMARY

Like other coronaviruses, infectious bronchitis virus (IBV) has a high mutation rate leading to the continuous emergence of variants. As a result, sequencing these variant strains using conventional PCR primers often fails. Oxford Nanopore (ONT) is a novel technology with the ability to produce long reads that could bridge highly variable sequences. In this study, we developed a new ONT sequencing workflow based on multiplex PCR tiling of the IBV genome to explore faster, simpler and less expensive IBV whole genome sequencing directly from clinical samples. The "Primal Scheme" primer design tool was used to design the multiplex PCR primer sets (n= 35) that cover >98% of the IBV genome with amplicon length = 1kbp. The newly developed nanopore workflow was then tested against five known positive IBV clinical samples. The generated amplicons were then sequenced using ONT Rabid barcoding kit RBK004. The new IBV ONT workflow could detect the IBV in the five tested samples as well as obtain most of the whole sequences of the IBV genomes even when the sample had a low C_T value (C_T=30). However, modification of the designed primers is still warranted in order to improve genome recovery using this approach.

INTRODUCTION

Despite circulating in the field for decades, IBV still challenges the poultry industry worldwide, leading to considerable economic losses1. The appropriate selection of protective live attenuated vaccines1 facilitates effective prevention and control of IBV. Therefore, the accurate and rapid identification and characterization of the circulating IBV strain is a critical step in this process2. Currently, genotypic classification of IBV commonly relies on serotype-specific S1 qRT-PCR3 or pan-IBV S1 RT-PCR assays coupled with Sanger sequencing1. Multiple factors impede these genotypic diagnostic methods, including the rapid evolution of the virus and the presence of multiple IBVs in the same sample4, 5, which accentuates the need for enhanced laboratory testing. ONT is a third-generation sequencing platform currently being adopted for the rapid diagnosis of avian pathogens6, 7. ONT is capable of producing long reads, which represents an opportunity to circumvent the need for S1-specific primers. A previous ONT workflow was developed for IBV5; however, the workflow was only targeting the S1 gene using traditional sanger sequencing primers and required considerable time and cost to be completed due to the use of ONT ligation sequencing kits. In this study, a new multiplex PCR-based protocol coupled with rapid ONT for IBV whole genome sequencing was developed and compared to the current sanger sequencing protocol to diagnose five IBV clinical submissions.

MATERIALS AND METHODS

In silico design of the multiplex PCR primer sets. The "Primal Scheme" primer design tool (https://primalscheme.com/) was used to design the IBV multiplex PCR primer sets covering the entire IBV genome8. The amplicon length was chosen to be 1 kbp. Primer design was based on 29 WGS of IBV representing different genotypes.

RNA extraction and Reverse transcription. RNA from five different clinical samples was performed using MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, the cDNA was generated using SuperScript IV (ThermoFisher Scientific).

Multiplex PCR amplification and quantification. Primers were resuspended at a concentration of 100 µM each and separated into two pools: 10 µL of each odd region primer was added to the Eppendorf tube labeled "Pool 1", and each even region primer was added to the Eppendorf tube labeled "Pool 2". Two multiplex PCR reactions were performed per sample for each of the two primer pools. After amplification, "Pool 1" and "Pool 2" were mixed

together in one tube per sample and quantified using Qubit 4 Fluorometer and High Sensitivity dsDNA assay kit (Thermo Fisher Scientific)

Nanopore library preparation. The amount of DNA for each sample was adjusted to 100 ng in 7.5 µL PCR grade water and barcoded individually following the ONT rapid barcoding (RBK004) protocol. The prepared library was loaded onto R.9.4.1 flow cell using the MinION device (ONT).

Data analysis. Guppy v5.0.11, under the control of MinKNOW 21.06.0. was used for base-calling of the Raw FAST5 files and demultiplexing of the barcodes. Genome coverage was estimated using the alignment of the samples against the complete genome of IBV prototype strain (Beaudette -M95169) retrieved from the GenBank. Detection and calculation of IBV sequencing reads were performed using ONT cloud-based pipeline EPI2ME-WIMP workflow (v3.5.7)

qPCR and Sanger sequencing. Identification of the IBV in the clinical samples was achieved by using universal qPCR targeting 5'UTR 9, while the classification of detected strains into the corresponding genotype was done using a panel of sanger sequencing primers targeting different regions of the S1 gene10-13

RESULTS

In this study, we introduced a versatile ONT sequencing protocol to detect and recover the whole genome sequence of IBV directly from clinical samples. Two sets of primers were designed to amplify the whole genome of IBV. Pool 1 consisted of 18 primer pairs and Pool 2 of 17 primers. The workflow was tested against clinical samples with C_T ranged between 18 to 30. The complete workflow is described in Figure 1. After sequencing of 5 samples for 6 hrs, the total number of generated bases was 377.12 Mb. Taxonomic classification revealed a high percentage of the reads were classified as IBV (Table 1). Whole genome coverage for different samples was >85%.

CONCLUSION

These results prove the clinical application of nanopore sequencing for the detection and genomic characterization of IBV directly from clinical samples compared to current diagnostics. Our hypothesis was the overlapping primer sets, in combination with the long reads of ONT, would allow for the primer-independent sequencing of the complete genome, including the full S1 gene. While the whole genome coverage from all tested samples was more than 85%, the S1 gene sequencing was still inconsistent. Modification of the designed primers is still warranted in order to improve S1 gene-generated sequences and genome coverage. Also, validation of the workflow against a large number of samples is still required.

ACKNOWLEDGMENT

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Table 1. CT values, number of generated reads and number of IBV-classified reads of the five tested samples

Sample	qPCR	Reads analyzed	IBV - Identification	# of IBV- reads	% of reads classified as IBV
1	30.63	66,370	Positive	53,538	94.8%
2	24.5	277,118	Positive	223,638	97.6%
3	25	42,926	Positive	34,852	98.1%
4	21.1	67,076	Positive	54,066	98.2%
5	18.67	123,414	Positive	92,042	95.2%

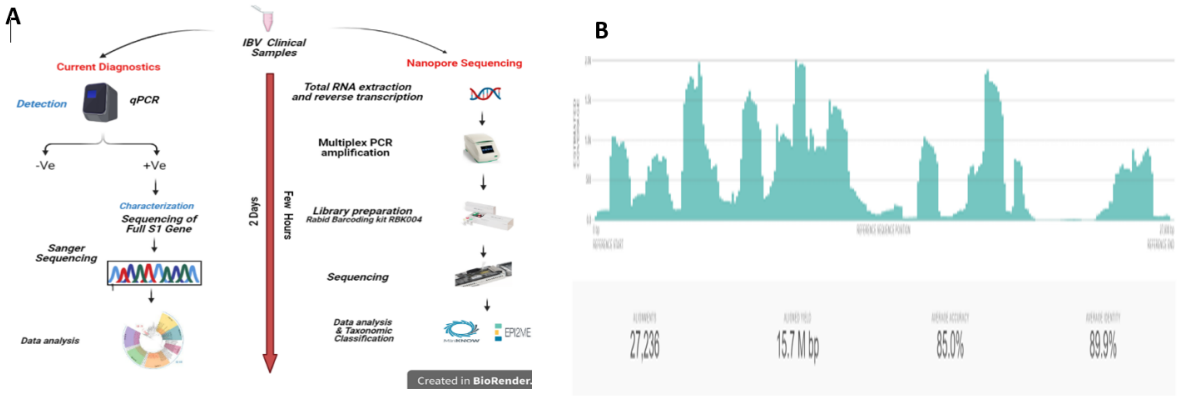

Figure 1: **A:** Schematic overview of the comparison between the current IBV molecular diagnostics and the new ONT workflow based on multiplex PCR tiling the WGS of IBV. **B**: Sequencing coverage and depth of sample 4. The vertical axis represents sequencing depth and the horizontal axis represents genome coverage.

META-ANALYSIS OF THE CHANGES IN THE PREDICTED METAGENOME FUNCTIONAL CONTENT AFTER INFECTION WITH COCCIDIA AND/OR *CLOSTRIDIUM PERFRINGENS*

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SUMMARY

Eimeria spp. infect cells in the intestinal mucosa of chickens. In addition to, and as a consequence of, the direct damage they cause, the infection leads to shifts in the intestinal microbiota. The most consequential change is a predisposition for a growth advantage to the bacterium Clostridium perfringens (CP), frequently leading to Necrotic Enteritis. Exploring these changes has been of interest for a long time because a better understanding is helpful in finding and evaluating prevention strategies. Recent technological advances to analyze microbial diversity by 16SrRNA next generation amplicon sequencing has enabled a large number of experiments investigating the topic in recent years. However, the results are inconsistent because of the large number of other factors that influence the composition of the intestinal microbiota. This meta-analysis summarized and analyzed experiments in which chickens were challenged with coccidia and/or CP.

Web of Science and PubMed were searched for articles reporting experiments involving infection with either one or both pathogens, in which the intestinal microbiota was analyzed by 16S rRNA next generation sequencing on the Illumina platform. Only experiments with results available as sequence data in public databases or from the authors were included. Sequences of all experiments were pre-processed in qiime2 using the same method to obtain Amplicon Sequence Variants and abundance tables. From them, the metagenomes of the communities were predicted, and pathway abundances were calculated in PICRUSt2. Frequency tables of pathways were imported into R. For each of the three datasets – infection with coccidia only, infection with CP only and infection with both pathogens – a random effects model without moderators was fitted using the metafor package.

Inclusion criteria were met by 17 experiments. Nine experiments had groups infected only with coccidia, four had groups infected only with CP, and eight had groups infected with a combination of both pathogens. In total, 834 samples were analyzed.

Relative abundance of 90 pathways in 16 classes was significantly changed after infection with coccidia only, of 111 pathways in 12 classes after infection with CP only and of 172 pathways in 19 classes after the combined infection. The infections with only coccidia mostly affected pathways related to cofactor carrier and vitamin metabolism, nucleoside and nucleotide metabolism, amino acid metabolism, and precursor carrier and vitamin metabolism. Infections with only CP mostly affected pathways related to amino acid metabolism, cofactor carrier and vitamin biosynthesis, nucleoside and nucleotide biosynthesis, and carbohydrate metabolism. The combined infections mostly affected pathways related to amino acid metabolism, nucleoside and nucleotide metabolism, cofactor carrier and vitamin metabolism and carbohydrate metabolism.

These results contrast with previous research that was focused mostly on short chain fatty acid metabolism. The scope of further research into the metabolomic changes after infection with coccidia or CP and interventions to mitigate the damage done by infections with these pathogens.

(The full-length article has been submitted to *Poultry Science*.)

2022 UNITED STATES HIGHLY PATHOGENIC AVIAN INFLUENZA OUTBREAK AND USDA RESPONSE

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SUMMARY

USDA, APHIS, VS, Mission. APHIS works in a variety of ways to protect and improve the health, quality, and marketability of our nation's animals, animal products and veterinary biologics.

As the Nation's veterinary authority, VS improves the health, productivity, and quality of life for animals and people, and maintains and promotes the safety and availability of animals, animal products, and veterinary biologics. VS integrates One Health principles with our USDA business objectives by contributing leadership, expertise, infrastructure, networks, and systems to collaborate effectively with local, State, Tribal, national, and international partners. Our comprehensive and integrated on-farm surveillance activities provide us the capability to achieve national goals for animal disease prevention, detection, and early response.

Goals of USDA high pathogenicity avian influenza (HPAI) response. The goals of an HPAI response are to:

1) Detect, control, and contain HPAI in poultry as quickly as possible.

2) Eradicate HPAI using strategies that seek to protect public health and the environment, and stabilize animal agriculture, the food supply, and the economy.

3) Provide science- and risk- based approaches and systems to facilitate continuity of business for non-infected animals and non-contaminated animal products.

Achieving these three goals will allow individual poultry facilities, States, Tribes, regions, and industries to resume normal production as rapidly as possible. The objective is to allow the United States to regain disease-free status without the response effort causing more disruption and damage than the disease outbreak itself.

USDA primary response strategy for HPAI. The United States' primary control and eradication strategy for HPAI in poultry, as recommended by the World Organization for Animal Health (WOAH), is "stamping-out." If the spread of HPAI outpaces the resources for stamping-out, or if other factors direct the response away from a stamping-out strategy alone, emergency vaccination strategies might be considered.

2022 U.S. H5N1 HPAI status. On January 13, 2022, the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) confirmed the presence of Eurasian H5 highly pathogenic avian influenza (HPAI) in samples collected in December of 2021 from a hunter harvested American wigeon in Colleton County, South Carolina. Additional confirmations in North Carolina and Virginia soon followed and by February 25, almost every State on the East Coast had reported HPAI.

On February 8, 2022, APHIS confirmed the first domestic HPAI detection in a commercial poultry flock in Indiana. APHIS immediately implemented its emergency HPAI plans, which included culling all poultry at the operation, creating a quarantine zone around the affected operation, and enhanced disease surveillance.

As of Friday December 9, 2022, the United States has confirmed 675 flocks - birds tested and confirmed having HPAI (287 commercial flocks and 388 backyard flocks) in 47 affected states; confirmed HPAI in 4,234 wild bird specimens across the U.S., including Alaska and Washington D.C., and depopulated over 53.02 million birds.

As of December 1, 2022, APHIS has committed over $372 million for indemnity for depopulated birds and eggs, as well as over $126 million for depopulation, disposal, and virus elimination activities. Current associated personnel, state agreements, and field costs are estimated at an additional $108 million.

Based on analysis of more than 5,729 full genome sequences and in consideration of epidemiologic data available to NVSL, as of November 29, 2022, at least 85% analyzed U.S. detections in poultry premises and non-poultry flocks are consistent with independent wild bird introductions. Phylogenetic support for potential lateral transmission or common source exposure has been identified in approximately 15% of events. Epidemiologic investigations have been launched. The National Veterinary Services Laboratories will continue to sequence all newly detected premises, analyze those results, and report them to appropriate officials.

There is significant risk of additional detections moving into the spring of 2023, at commercial operations and backyard premises due to wild bird migration patterns. USDA APHIS has recommended increased precautions by poultry producers to prevent flock exposure to wild birds; developed a timeline for depopulation, disposal and virus elimination and change the way payment is made for compost litter; lowered the post-virus elimination environmental

testing requirement number per barn from 10 samples to 5 samples, reducing the drain on resources; revised 150-day fallow period to 120 days as a virus elimination tactic in non-poultry (backyard flock) premises. This reduction is sufficient to allow virus degradation in typical farm substrates and environmental conditions. These new policies will help streamline future outbreak responses.

My presentation will focus on the 2022 United States highly pathogenic avian influenza outbreak and USDA Response.

THE USE OF MONOGLYCERIDES AND MONOGLYCERIDE BLENDS FOR MANAGING GUT HEALTH

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SUMMARY

The increase in consumer activism regarding the use of antibiotics in poultry production drove a seismic shift in coccidiosis control programs and the industry approach towards gut health. In 2014, only 3% of the United States broiler industry raised birds in a No Antibiotics Ever (NAE) program with the remainder using the more traditional, ionophore-based programs with or without the addition of antibiotic growth promoters. Compare that to 2020 where those numbers have flipped: 58% of broilers are raised NAE, 40% are raised without antibiotics medically important to human medicine and only 2% are raised conventionally.

The loss of not only the anticoccidial benefits of ionophores but probably even more consequentially, the loss of the anti-inflammatory properties of ionophores (1), resulted in a steep increase in subclinical and clinical necrotic enteritis (NE) and the resulting loss of performance and increase in mortality. To fill that gap and restore intestinal integrity and function, the industry turned to a number of feed additives to reduce the inflammation of digestion and coccidiosis cycling and to suppress Clostridium perfringens. The usage of enzymes, saponins and other emulsifiers, organic acids, monoglyceride ester blends, phytogenic compounds, and others have become commonplace. Feed additives have gone from being the sole purview of the nutritionist to essential items in the veterinarian's toolbox.

In this article, we will discuss the antimicrobial properties of monoglycerides, their systemic and local effects on inflammation and immunity, along with their impacts on adipose deposition and carcass yield.

Improvement of Organic Acids by Conversion to Monoglycerides. Organic acids/fatty acids have long been understood to impart gut health and pathogen control benefits and have been deployed in a variety of forms and combinations in both feed and drinking water for some time. Organic acid usage at levels necessary for consistent efficacy come with many drawbacks, most notably corrosivity, odor and palatability concerns. To achieve consistent Salmonella control with organic acids, especially single acids rather than blends, the veterinarian finds themself balancing effective inclusion rates with ones that will not reduce feed or water consumption.

A change in form, by means of a chemical reaction with glycerol to transform an organic acid into a monoglyceride or monoacylglycerol (MAG), allows for the function of organic acids to be maintained and often improved while removing the corrosivity, odor and palatability issues that previously limited their use. Butyric acid is the most classic example with its vomit-like odor that is so oppressive to feedmill employees. Converting butyric acid into a butyrate glyceride (C4), such as monobutyrin or tributyrin, eliminates the odor that previously restricted its use while at the same time enhancing its function by protecting the butyrate parent molecule from rapid absorption in the upper GI tract. Butyric acid is mostly fat-soluble in either its uncoated or coated form whereas monobutyrin becomes mostly water soluble. The shift in monobutyrin solubility allows for both delivery into the lumen, where it supports the health of the enterocytes, but also into the portal circulation by absorption along with water in the hindgut where its systemic anti-inflammatory properties can be realized.

MAGs of short-chain (<6 carbons) and medium-chain (7-12 carbons) fatty acids (SCFA and MCFA, respectively) are often discussed as a single, monolithic class imparting class-wide benefits, whereas in reality MAGs have activities specific to their parent molecule and carbon chain lengths. Therefore, not all MAGs impart the same benefits to the bird. Tributyrin is a triglyceride that is metabolized through the actions of endogenous lipase (2) into free butyric acid and monobutyrin, but for simplicity's sake will be discussed here with the other MAGs. Monobutyrin is active in the bird as consumed whereas tributyrin is not biologically functional until it is broken down into the active forms of monobutyrin and butyric acid.

Fatty acids are amphiphilic molecules. The carboxyl group of the fatty acid is hydrophilic, and the carbon chain is hydrophobic allowing them to serve as an interface between polar and non-polar liquids in order to decrease surface tension providing emulsification benefits in the diet. The aqueous solubility of the fatty acid is determined by its carbon chain length and in general, the longer the carbon chain, the less water-soluble the molecule (3). Unlike free fatty acids which lose their hydrogen ion (H+) becoming anionic around the physiologic pH of 7.4, MAGs do not have ionizable function groups at biologically relevant pHs and therefore are neutrally charged across a wide range of pHs allowing for biological activity across the entire gastrointestinal tract (4).

Antimicrobial Lipids. The amphiphilic properties of the FAs and MAGs which destabilize bacterial cell membranes earns them the moniker "antimicrobial lipids (4)." As such, naturally occurring fatty acids are integral components to the innate immune function of the skin. The antimicrobial properties of MAGs were first reported by Lamar (5) in 1911 in studies with Pneumococcus but were quickly overshadowed by the dawn of the antibiotic era around that same time. One could argue that the poultry industry is now entering a "post-antibiotic" era and that MAGs are finally getting their due. One of the advantages of antimicrobial lipids, whether in the form of free fatty acids (FFAs) or MAGs is the difficulty bacteria have in developing resistance mechanisms. Because of this, bacteria can be grown in sub-lethal concentrations of antimicrobial lipids for at least one year without developing any signs of bacterial resistance (6) allowing the veterinarian to confidently deploy them widely in a commercial production setting.

Not all MAGs have the same mode of action against bacteria. Some, such as monolaurate (C12) and monocaprin (C10), destabilize bacterial lipid bilayers through tubule formation, complete solubilization of the lipid bilayer or both. Others, such as monocaprylate (C8) integrate into the pore structure of the cell membrane causing increased permeability and membrane fluidity. The innate fluidity and phospholipid composition of the target organism determine which bacterial strains are most sensitive to which MAG (3). These bactericidal effects also require the MAGs to be present in concentrations at or above the critical micelle concentration or CMC. At concentrations below the CMC, FAs and MAGs may still have some bacteriostatic effect on the membrane thereby inhibiting cell division (3) or they may have no activity at all depending on the compound and the target organism. Along with the permeability disruptions to the bacterial cell membranes, MAGs also interfere with the electron transport chain and oxidative phosphorylation along with direct inhibition of membrane enzymes interfering with nutrient uptake (4).

Micelles are the biologically active structures of medium-chain monoglycerides (MCMG) which can only self-assemble when above the critical micelle concentration of their respective compounds or blends. The lower the CMC, the more easily micelles are formed and the lower the concentration or dietary inclusion rate necessary to observe clinical benefit. The exception to this rule would be monocaprin (C10) which begins to induce tubule formation at concentrations slightly below its CMC. MCMGs that have longer chains (C8-C12) have lower CMCs due to their better self-assembly of micelles. This also explains why most MCMGs are more potent antimicrobials than their corresponding FFAs. Because they are non-ionic, MCMGs form micelles more easily and have lower CMCs than their charged counterparts (7).

It is these structure-to-function relationships that explain why certain MAGs are more antimicrobially potent than others as well as explaining why some target organisms are more sensitive to certain MCMGs than others. For example, lauric acid (C12) has the longest carbon chain among the MCFAs and the lowest CMC of the MCFAs explaining why it is the most antimicrobially potent. However, when transformed into glycerol monolaurate (GML), the antimicrobial potency is increased exponentially because micelles of GML are self-assembled more easily than those of lauric acid. This explains why the minimum bactericidal concentration (MBC) of GML against S. aureus is 0.25μM as compared to 50μM for its parent molecule lauric acid (6).

As mentioned previously, there is a bit of a hand-and-glove effect in terms of antimicrobial efficacy with some MCFAs and MCMGs working better on specific target organisms compared to others, and with activity against Gram-positive bacteria usually better than that against Gram-negatives. In addition, a significant amount of cross-study variation exists in terms of efficacy so empirical testing of clinical isolates can be useful in determining product choice. Generally, lauric acid and GML have been shown to have the broadest and most efficacious antibacterial activity, showing efficacy against Listeria monocytogenes, Clostridium perfringens and Staph. aureus (4). Monocaprin (C10) has been shown in multiple studies to have efficacy against Campylobacter jejuni through both feed and water delivery, though inhibition in commercial settings can be variable due to mucus layer interference (9, 10, 11,12). Bunkova et al (13), also showed that monocaprin had good bactericidal efficacy in vitro against *Salmonella* and *E. coli*. Caprylic acid and monocaprylate (C8) have shown the best efficacy against *E. coli* (12) and *Salmonella* Enteriditis (15, 16) generally. A blend of monocaprylate and monocaprin has also shown efficacy against *Salmonella* Typhimurium and *E. coli* in a separate study by Neath et al (17). Short-chain fatty acids such as formic, propionic, and butyric acids also exhibit clinical benefit in reducing Salmonella numbers in poultry and have been used for decades in commercial poultry production for such purposes. It should be noted that triglycerides such as tributyrin are typically not antimicrobial as consumed but must be converted by lipase into more active forms (7).

Enveloped viruses such as avian influenza, infectious bronchitis virus, African swine fever virus and porcine epidemic diarrhea virus are also susceptible to membrane disruption by MCMGs. These compounds are now routinely used to inactivate infectious viruses present in swine feeds, for example. An interesting study published by DeGussem et al (18) evaluated the impact of a feed-delivered GML (6-lbs/ton) on immune response to Infectious Bronchitis (IBV) vaccination. The study was initially conducted because of concerns that the disruption of viral envelopes from

the feeding of GML might impede the immune response to IBV vaccination. In fact, the opposite occurred with birds fed the GML having double the antibody titers and faster vaccine clearance than the untreated birds. It is unclear if the improved vaccination response was due to direct GML effects on the vaccine virus and how it was presented to the immune system or indirectly due to enhanced systemic immune benefits of GML. Further study is clearly warranted to see if feeding of MCMGs can enhance other live virus vaccination of broilers or live priming in breeder programs.

Immune System Regulation and Gut integrity. Though the toxin production of *Clostridium perfringens* (CP) is primarily thought of as the main player in the development of necrotic enteritis, it is the T-cell mediated inflammatory response to coccidiosis (primarily Eimeria maxima) that truly sets the stage. One can argue that without that inflammation, CP would be of little consequence. Inflammation of the intestinal mucosa can also occur from feed ingredients and be thought of as the "inflammation of digestion" that occurs even in the absence of a significant cocci challenge.

Butyric acid and its derivative monobutyrin impact the clinical expression of necrotic enteritis most likely due to its anti-inflammatory properties and impact on epithelial repair rather than any direct impact on CP. Interestingly, butyrate and its glycerol derivatives are routinely used in human medicine as luminal infusions post-intestinal anastomosis because their epithelial proliferation and support properties speed up wound healing. Monocaprylate also shows significant anti-inflammatory effects both locally and systemically through upregulation of anti-inflammatory cytokines and down regulation of pro-inflammatory cytokines (19). These anti-inflammatory benefits of MAGs cannot be separated from the promotion of intestinal integrity and the maintenance of homeostasis.

A variety of local and systemic immune responses occur with dietary supplementation of both SCFA, MCFAs and their corresponding MAGs. Tight junction protein 1 (TJP1) serves as a proxy for all tight junction genes and has been shown to be upregulated in broilers by supplementation of a blend of monobutyrin, monocaprylate and monocaprin at 10-lbs/ton (20) along with an increased systemic immune response.

It is hypothesized that the majority of the immune response exhibited in influence from fatty acids and MAGs is through the activation of free fatty acid receptors (FFAR). FFAR 1 and 4 are activated by MCFAs while FFAR 2 and 3 are activated by SCFAs, most notably butyric acid and monobutyrin (21). FFAR2s are found in gastric immune cells, neutrophils, eosinophils, and monocytes along with intestinal T-reg cells. FFAR2 regulates inflammatory processes in the gut and controls epithelial integrity. The anti-inflammatory effects of butyrate and monobutyrin were confirmed to be because of the binding to FFAR2 by the use of knock-out mice who had this gene deleted (22).

From a clinical perspective, these anti-inflammatory properties are manifested in the substantive reduction in NE-induced mortality under various challenge models. Both Kumar (20) and Liu (unpublished) demonstrated treatment associated mortality differences in a NE-challenge model using two different commercially available monobutyrin, monocaprylate and monocaprin blends. The blend used in the Kumar study was efficacious at an inclusion rate of 10-lbs./ton while the blend tested by Liu showed a clinically significant decrease in NE mortality at 4-lbs/ton.

Carcass Quality and Yield. While the antimicrobial and immune-supportive properties of MAGs are of greatest interest to the clinical veterinarian, it is the carcass yield uniformity benefits that provide the greatest interest to the poultry integrator. Makowski et al (23) showed that supplementation with monobutyrin at 7.5-lbs/ton in turkeys increased breast yield by 1.3% by shifting the bird towards protein synthesis and away from fat deposition. Monobutyrin-treated birds were 0.5 lbs. heavier at 15-weeks with a 7-point reduction in feed conversion and a 1% increase in livability. Multiple studies with a Canadian research group (24, 25, 26, 27) also confirmed that varying levels of monobutyrin blends (2-12-lbs/ton) showed increases in either breast yield, reduction of abdominal fat deposition or both. This shift occurred due to the shift in lipid catabolism in response to the presence of monobutyrin (23).

Strategies for Use of MAGs in Live Production. The challenge for the clinical veterinarian is how to harness the physiological benefits of MAGs into programs that address specific clinical or production needs, balancing inclusion rates high enough to see clinical benefit but also be able to pay for themselves in return.

Butyrate glycerides should be chosen when looking for anti-inflammatory and intestinal integrity benefits as well as for increased carcass yields, while butyric acid and its salts can be effective in mitigating Salmonella. MCFA and their MCMGs are the class of choice for antimicrobial properties. Monocaprylate is best chosen when seeking to control Gram-negative bacteria such as *Salmonella* and *E. coli* while monocaprin, particularly delivered via drinking water, is best suited for Campylobacter control. Lauric acid and GML show the greatest efficacy towards Gram-positive bacteria. The key to all MCFAs/MCMGs will be using them at high enough inclusion rates in order to exceed the CMC so that they can be in the proper micellular form necessary to disrupt bacterial and viral cell membranes. The MCMGs will also impart some anti-inflammatory benefit through mitigation of cytokine responses. Thankfully, synergies exist between MAGs and SCFA/MCFAs that may allow for lower inclusion rates than when using single

ingredients alone. The use of liquid concentrates of MAGs rather than dry carriers will also allow for lower inclusion rates for feed-delivered products with increased concentrations that are more economical.

SUMMARY

Short and medium-chain fatty acids and their respective monoglycerides used at sufficient dietary inclusion rates show positive anti-inflammatory, antimicrobial, and intestinal integrity benefits. Monoglycerides and their blends are clinically relevant tools for the veterinarian to support bird health and performance.

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Desired Benefit/ Target Organism	**Ingredient Choice**
Salmonella	Butyric acid and monocaprylate/monocaprin blends
Campylobacter	Monocaprin and monocaprin blends
Gram (+) bacteria	Lauric acid and glycerol monolaurate
Reduced Inflammation	Monobutyrin and monocaprylate blends
Carcass Yield	Monobutyrin and monobutyrin blends

EPIDEMIOLOGY AND CONTROL OF AVIAN INFLUENZA IN INDONESIA – INDUSTRY PERSPECTIVE

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SUMMARY

AIV has been circulating in Indonesia for 20 years. The government policy is to prevent disease by vaccination. Vaccination can reduce AI cases, clinical symptoms, and mortality but not the emergence of new viruses. These are partly due to the discrepancy in vaccination practice in different poultry sectors.

Rapid detection of the changing field viruses is the key to creating vaccines with good protection. Our surveillance program showed that H5N1 2.1 circulated from 2003 to 2016, then replaced by H5N1 2.3.2.1c in 2012, and in 2019 two patterns of 2.3.2.1c were detected. H5N1 2.3.4.4b was recently found. H9N2 H9.4.2.5 was isolated from 2016 until now. Chickens without H9N2 vaccination could be the predisposition to recurrent outbreaks of H5N1. Homology of vaccine isolates contributes to protection. Recent government policy to permit the use of isolates from independent surveillance in vaccine may incentivize industries to strengthen their surveillance programs and allow faster update of vaccine isolates.

INTRODUCTION

Avian influenza has been circulating in Indonesia since late 2003. The firstly introduced H5N1 virus was found in 9 provinces which then triggered the policy for a nationwide vaccination program. The H9N2 viruses were detected later in 2016. The vaccine isolates for both H5N1 and H9N2 were determined and provided by the government. Vaccination is estimated to cost USD 169 per rearing period.

The poultry industry in Indonesia is classified into four sectors based on the level of biosecurity. The lack of biosecurity and vaccination in sector 3 and 4 might contribute to the changing field viruses and outbreaks. In this study, we presented the surveillance result of H5N1 and H9N2 from the year 2019 to 2022.

MATERIALS AND METHODS

Samples from chickens with clinical signs and pathological characteristics of AI were collected from all poultry sectors and live bird markets. The serum of AI-suspected chickens was tested with the HI test. The virus was isolated from organ samples by propagation in SPF eggs, continued by PCR and sequencing of HA and NA segments. The sequence data were analyzed based on phylogenetic tree results and homology.

RESULTS

The surveillance result showed the wide distribution of HPAI H5N1 and LPAI H9N2 viruses throughout Indonesia. Two patterns of H5N1 2.3.2.1c were found in 2019. The homology between the two patterns was 95.19 - 96.52% and show minimum cross-protection. The same H9N2 from clade 9.4.2.5 was found in 2019-2022 as the years before. The newly emerging H5N1 2.3.4.4b was detected in late 2022 from sector 3 and 4 in South Kalimantan.

The average peak period of AI was from January to May for H5N1 and from September to November for H9N2. Phylogenetic tree results showed the different grouping of H5N1 2.3.2.1c pattern 1 and 2 as well as 2.3.4.4b. The majority of field viruses were H5N1 clade 2.3.2.1c pattern 1 and H9N2 clade 9.4.2.5 Y280 lineage.

The prominent clinical signs of H5N1 were lethargy and death at >2 DPC for 2.3.2.1c and >4 DPC for 2.3.4.4b, while H9N2 showed deteriorated egg quality and decreased egg production. The pathological signs include colon hemorrhage and epicardial petechiae for H5N1; oviducts full of egg yolk for H9N2.

DISCUSSION

H5N1 2.1 viruses were detected from 2003 to 2016 and then developed into sub-clade 2.1.3.1, 2.1.3.2 and 2.1.3.3. Clade 2.1 viruses, last found in 2016, were replaced by viruses from clade 2.3.2.1c since 2012 until now with two

patterns detected. H5N1 2.3.4.4b viruses were recently found last year. H9N2 H9.4.2.5 viruses were circulating since 2016 until now.

Isolates for vaccines and the viruses for challenge tests were determined by Indonesian government based on the circulating virus in the field. The government provided H5N1 viruses for vaccine seeds since 2003. There was an attempt to use the vaccine isolate from H5N2 virus for DIVA in 2006 but it failed to provide sufficient protection. Since then, only homologous viruses were used in vaccines. In 2019, viruses isolated from independent surveillance were allowed to be used in the vaccine. The new policy may incentivize industries to strengthen their surveillance programs and allow faster updates of vaccine isolates.

Internal data revealed that chickens without H9N2 vaccination could be the predisposition to recurrent outbreaks of H5N1. With a previous outbreak of H9N2 in July 2018 resulted in both H5N1 and H9N2 outbreaks in 2020.

CONCLUSION

Indonesia is one of the countries that implement the vaccination policy. It is very crucial to match the circulating field viruses with vaccine isolates. Both H5N1 and H9N2 must be included in vaccines along with strict biosecurity and surveillance programs. Vaccination must be implemented in all sectors, including sector 3 and 4 as well as migratory birds. There is a warning of the new emerging H5N1 2.3.4.4b that starts to spread to other areas in Indonesia. Fast action must be taken to vaccinate chickens with this new virus to prevent or at least limit the spread.

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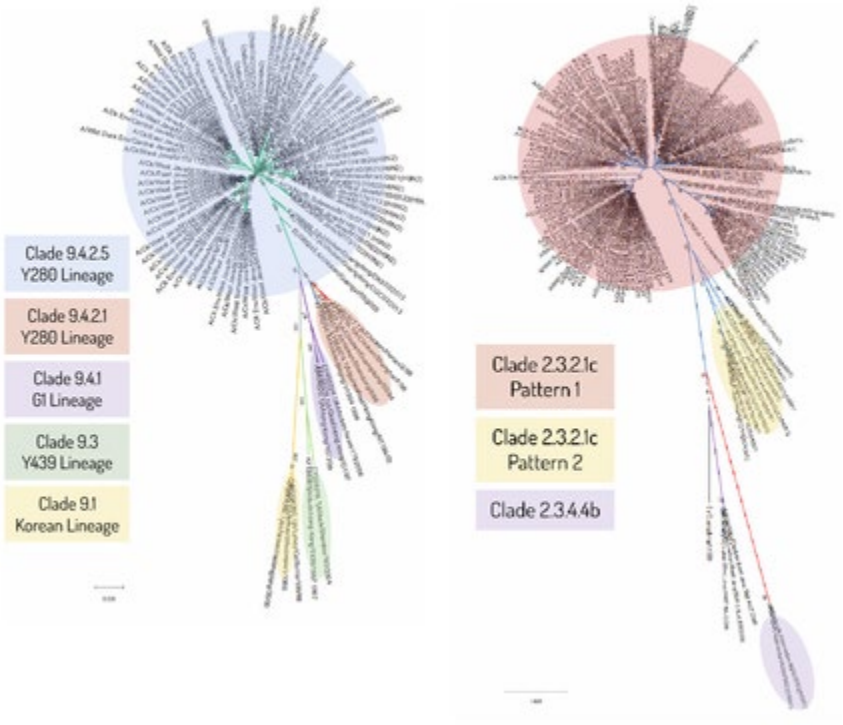
Figure 1. Geographical Distribution of AI Viruses in Indonesia from 2019 to 2022

Figure 2. Phylogenetic Analysis of H9N2 and H5N1 Viruses in Indonesia from 2019-2022



MICROSCOPIC PATHOLOGY ASSOCIATED WITH EARLY IBV VACCINATION AND CHALLENGE IN LAYING HENS

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SUMMARY

Infectious bronchitis virus, a gammacoronavirus primarily causing acute respiratory disease in chickens, has also been associated with development of urogenital disorders such as nephritis or false layer syndrome. The effects of passive immunity on development of chronic microscopic lesions in mature layers receiving vaccination and challenge within the first days of life has not been discussed. Here, we assess differences in tracheal epithelial thickness, deciliation, and lymphocytic infiltration, as well as presence of chronic microscopic lesions in kidneys and oviducts in mature layers, with and without maternal antibodies at hatch, after 30 weeks post-challenge and clearance of the virus. These analyses will further the understanding of the protective effect of maternal antibodies after early challenge and/or vaccination with IBV.

INTRODUCTION

Infectious bronchitis is an economically relevant endemic disease caused by the gammacoronavirus infectious bronchitis virus (IBV). While IBV primarily results in acute respiratory disease in chickens, the virus has also been implicated in the development of false layer syndrome (FLS) wherein hens will ovulate normally but be unable to produce eggs. FLS has been known to occur when IBV is introduced within the first days of life (1, 2). However, little investigation has been performed regarding assessment of the effect of maternal antibodies on the protection against IBV infection at these early ages which can lead to FLS inducing lesions. We have previously reported that maternal antibodies both dampen acute respiratory symptoms and reduce the incidence of gross lesions, such as cystic oviducts, oviduct atrophy, and yolk peritonitis, associated with false layer syndrome after early GA-type vaccination and DMV/1639 challenge. In a continuation of this investigation, we now aim to assess the long-term protection conferred by maternal antibodies on the microscopic tissue level in the tracheas, kidneys, and oviducts.

MATERIALS AND METHODS

Experimental design. Female SPF (n=120) and commercial (n=120) chicks were hatched at the UC Davis avian hatchery. Commercial eggs were sourced from a farm with a history of IBV vaccination. Day-of-age blood collection was performed to evaluate maternal antibody presence via IBV ELISA (IDEXX). Additionally, at day-of-age half of the birds from both SPF and commercial groups were administered 50µL of a GA-type live vaccine ($10^{6.8}$ viral RNA copies, measured by RT-qPCR targeting M gene) via the ocular route. At three days of age, the four groups were further divided in half and each half was administered 50µL of infectious bronchitis strain DMV/1639 ($10^{3.5}$ EID_{50}, $10^{5.7}$ viral RNA copies) via the ocular-nasal route. This resulted in 4 groups each of SPF and commercial birds: negative controls, vaccinated, challenged, and combined vaccinated plus challenged. Birds were raised to thirty-weeks of age, necropsied, gross pathology observed, and tracheas, kidneys, and oviducts collected for histopathology. Note, this experiment also included early investigation of respiratory signs and viral loads, documentation of egg production throughout life, and evidence of gross lesions related to false layer syndrome which have been previously presented and will only briefly be reviewed in the context of relation to histological findings.

Histopathology and histomorphometry. Tracheas, kidneys, and oviducts were collected from birds at 30-weeks of age, fixed in 10% formalin, sectioned transversally, and processed into paraffin-embedded blocks. Imbedded tissues were further sectioned at 4 microns and stained with H&E (hematoxylin and eosin) dyes. Organs were examined at 400X magnification by light microscopy for the purposes of histomorphometry. Tracheal and oviductal histomorphometry were performed similarly by measuring the thickness of the mucosal epithelium in sections of either organ as previously described (3). Additionally, sections of the magnum of the oviducts were scored 0-4 for lesions using the following criteria: 0 = no discernible microscopic lesions, 1 = loss of secretory albuminous granules, minimal inflammation, and/or minimal lymphoplasmacytic infiltration, 2 = loss of secretory glands, 3 = loss of

secretory glands in addition to edema and/or inflammation, and 4 = compression of the mucosal epithelium. Finally, kidney lesions were evaluated as previously described (3) and birds were considered affected if at least one lesion type was observed.

RESULTS

Oviduct histomorphometry. Figure 1 (A and B) illustrate the oviduct histomorphometry results in terms of mucosal epithelial thickness and lesions scores, respectively. There were no significant differences between oviduct epithelial thickness within either SPF or commercial bird groups, regardless of treatment. However, for each treatment assessed individually, commercial birds had thicker epitheliums of the oviduct mucosa compared to the SPF birds, significantly so for the control, vaccine, and combined vaccine and challenge groups. There were no significant differences in oviduct lesions scores amongst commercial birds. However, in the SPF group, which did not have maternal antibody protection, the lesion scores for IBV dosed birds were all higher than those scores seen in the control groups, significantly so for the SPF challenged birds.

Tracheal histomorphometry. There were no significant findings in terms of changes in tracheal epithelial thickness regardless of presence of maternal antibodies or treatment. No marked deciliation or lymphocytic infiltration was observed in any treatment group.

DISCUSSION AND CONCLUSION

Logically, the histopathological analysis of mature oviducts from birds infected at early ages with infectious bronchitis virus yielded the most interesting results. Given the control commercial birds had significantly higher epithelial thickness measures like the commercial vaccine and combined vaccine and challenge group, it can be concluded that epithelial thickness of the magnal portion of the oviduct is, overall, not affected long-term. Commercial birds merely have increased epithelial thickness in comparison to the SPF birds, likely a result of the genetic makeup of production birds. Conversely, given the results, there is potential that the administration of vaccine in conjunction with passive immunity could influence the retention of thicker oviduct mucosal epithelium in the event of early challenge. This seems to be contradictory to previous reports that maternal antibodies will prevent the induction of vaccine protection if vaccines are administered while passive immunity is robust (4) and may indicate further investigation into the interaction of vaccines and maternal protection is necessary. Microscopic lesion scoring of the oviducts was consistent with the gross pathology results reported earlier from this study, where the SPF birds with vaccine, challenge, or a combination treatment had higher scores than control birds, reaching significance in the challenge group. The fact that commercial birds, which had maternal antibody protection, did not have significantly higher lesions scores when dosed with IBV further supports that passive immunity is massively important in the protection of naïve birds. It is no surprise that tracheal epithelial thickness appeared unaffected by the long-term effects of early IBV infection given that respiratory presentation of the disease resolves quickly and regeneration at the microscopic level has been observed just a few weeks post infection (5). Kidney lesions have only minimally been assessed thus far. However, given the gross pathological findings of predominantly healthy kidneys, we do not anticipate kidney lesion scoring to yield significantly interesting results. In conclusion, it appears that long-term microscopic effects of early IBV infection were largely confined to the reproductive tract and our previous results that maternal antibodies protect against lesion formation when IBV challenge or live IBV vaccines are used is further supported.

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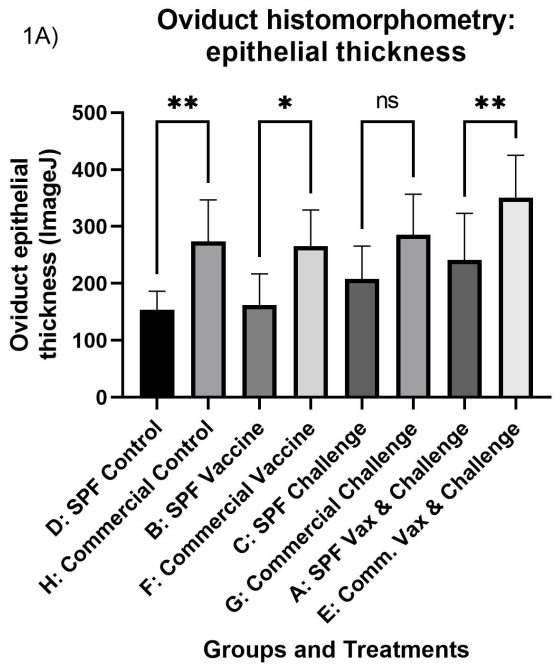
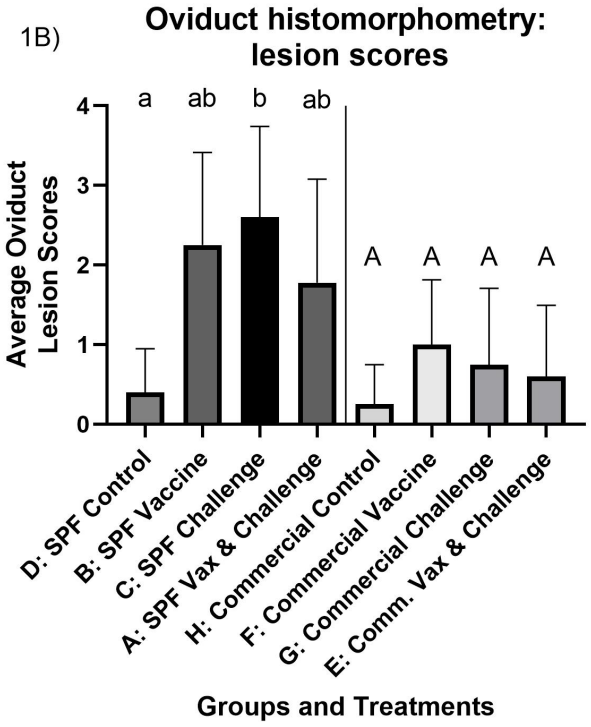
Figure 1. Oviduct histomorphometry comparing mucosal epithelium thickness (A) and lesion scoring of the magnum (B) between commercial and SPF birds with no treatment, vaccination, challenge or a combination. Significance in (A) is shown between SPF and control birds of the same treatment; ns: P>0.05, *: P≤0.05, **: P≤0.01. Significance in (B) is indicated by different letters, lowercase for SPF groups and uppercase for commercial groups.



1B)


Oviduct histomorphometry:
lesion scores
Average Oviduct
Lesion Scores
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D: SPF Control
B: SPF Vaccine
C: SPF Challenge
A: SPF Vax & Challenge
H: Commercial Control
F: Commercial Vaccine
G: Commercial Challenge
E: Comm. Vax & Challenge
Groups and Treatments

ANALYSIS OF VIRUS DETECTION FROM BROILER DIAGNOSTIC CASES WITH BURSAL ATROPHY

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SUMMARY

In this study, we investigate the viral etiology from diagnostic broiler tissue samples from the atrophic bursae of Fabricius submitted for the detection of infectious bursal disease virus (IBDV). Of these, IBDV-specific sequences were detected in only 45% of cases. All the sequences shared >97% amino acid identity with GA198, a variant of the Del E strain. Interestingly, a few other virus groups were also detected. Avian reoviruses were phylogenetically related to the isolates from viral arthritis and tenosynovitis cases reported from several states in the US since 2012. Fowl adenoviruses were also isolated from inclusion body hepatitis cases. Finally, regardless of the presence of IBDV, most bursal tissue extracts were positive for parvovirus-specific sequences. IBDV-suggestive histopathological lesions (necrosis) were detected in all the samples that were positive for GA-198. On the other hand, reovirus, adenovirus, and parvovirus-positive bursal tissues showed marked hypoplasia. These findings will help clinicians to consider other virus groups that can cause bursal atrophy and immunosuppression.

INTRODUCTION

The poultry house environment sometimes exposes chickens to immunosuppressive stressors and infectious agents that adversely affect innate and acquired immunity and diminish genetic and nutritional potential (1). Amongst infectious agents, IBDV, Marek's disease virus (MDV), chicken anemia virus, and mycotoxins are some of the important immunosuppressive agents that cause marked bursal atrophy. Several other viral agents including avian reovirus, fowl adenovirus (FAdV), Newcastle disease, infectious bronchitis, and avian parvoviruses (PV) have been shown to cause bursal atrophy (2). IBDV infection frequently results in other opportunistic infections like reoviruses (ARV) and PV.

During the investigation of the atrophic bursal tissues submitted for the detection of IBDV over a 4-year period (2019-2022), we observed that only 38 out of 84 tissues were positive for IBDV. Histopathology of bursal tissues from non-IBDV cases showed varying degrees of bursal atrophy due to lymphoid depletion or hypoplasia, but no necrosis. Many affected chickens showed signs of inclusion body hepatitis (IBH, caused by FAdV subtypes), viral arthritis, uneven growth, enteritis, and respiratory problems. The purpose of the current study is to detect the viral agents from the IBDV-negative bursae from chickens showing bursal atrophy. We also sought to investigate concurrent infections, if any, from bursal tissues.

METHODS

Viral RNA Isolation and RT-PCR. Bursae were homogenized in phosphate-buffered saline supplemented with antibiotics. Crude virus suspension was clarified by centrifugation and used for virus isolation and viral RNA extraction. For the isolation of ARV and FAdV), monolayers of chicken embryo kidney cells were inoculated with crude virus suspension. After three passages, the cytopathic effect in primary chicken embryo kidney cultures (CEK) was characterized by the formation of syncytia within 48 to 72 hours after inoculation for ARV, whereas FAdV caused marked rounding and cell detachment. Supernatants from CEKs were aliquoted and stored at -80C.

Viral RNA was extracted using Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. The amplification of the VP2 gene segment IBDV was performed using a SuperScript III OneStop RT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA) as described previously (3). For the detection of ARV, the amplification of the sigma C gene was performed using previously described primers (4).

DNA isolation. Total DNA extracted from the clarified supernatants of bursal homogenate using QIAmpDNA Mini kit (Qiagen, Valencia, CA). For the detection of FAdV, a LongAmp PCR kit (NEB, Ipswich, MA) and previously described hexone-specific primers were used (5). Amplification of a PV-specific NS gene segment was amplified as described by Zsak et al (6).

The PCR products of IBDV, ARV, FAdV, and PV (approximately 525, 900, 1000, and 560 bp, respectively), were purified using QIAuick PCR purification kit (Qiagen, Carlsbad, CA), and sequenced using the forward primer of the respective amplicon. The sequencing reaction was performed using the BigDye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA) on ABI 3730xl DNA Analyzer at the Azenta genomic facility (South Plainfield, NJ).

Phylogenetic analysis. Sequence information was compiled with the SeqMan program (Lasergene v15.0, DNASTAR, Madison, WI). The alignment of sequence data and the construction of the phylogenetic tree was performed by the MegAlign program (v15.0, DNASTAR) using the Clustal W multiple sequence alignment algorithm as described earlier (7). The ARV reference strains from the US that showed high similarity in the basic local alignment search tool (BLAST) were selected for analysis.

RESULTS

Clinical history. Bursal tissues from cases showing marked bursal atrophy were submitted for the detection of IBDV. Affected chickens also showed signs of inclusion body hepatitis, stunted growth, viral enteritis, and leg problems. Several other tissues were submitted for the detection of FAdV as well as ARV from the affected organs. Of the eighty-four tissues submitted for IBDV detection, only 38 were positive for IBDV. The median age of IBDV-positive and IBDV-negative cases was 27 and 19 days, respectively.

Virus isolation/detection profile from bursal tissues. Thirty-eight out of 84 tissues were positive for IBDV. Mixed infections due to FAdV, PV, and ARV were observed in 14, 13, and 6 samples, respectively (Figure 1A). Amongst the remaining forty-six IBDV-negative tissues, ARV was the most predominant followed by FAdV and PV. A mixed infection of ARV with PV and IBHV was also observed in this group (Figure 1B).

Phylogenetic analysis of IBDV nucleotide (nt) sequence. Thirty-eight out of 84 bursal tissues were positive for IBDV. The VP2 sequence similarities were confirmed by using the neighbor-joining method. All the fourteen sequences included in the analysis were 97 to 99 percent identical to GA198. All these sequences were grouped with the E/Del sequence, with a nodal value of 95, which showed a high probability of that being the true location of the sequence (Figure 2).

Phylogenetic analysis of the ARV isolates from bursal tissue. ARV was frequently isolated from IBDV-negative bursal tissues. For the molecular analysis of the S1 segment, sequences from 13 representative sequences from the bursal tissues, nine isolates from 2011-2015 cases of viral arthritis, and six ARV reference sequences from GenBank were included. All the isolates were grouped into four distinct phylogenetic groups as described previously (8,9, Figure 3). Of the 13 field isolates, seven were in the same cluster (Group 2) as the variants reported elsewhere in Georgia (10). All these isolates showed 95 to 100% nt similarity to 2010 isolate JX983602 from broilers with runting-stunting cases. Interestingly, several isolates from viral arthritis cases were also grouped with bursal isolates. Four out of 13 isolates were grouped (Group 1) with those from 2013-2015, and 2017 cases of tenosynovitis from GA, PA, and CA. Finally, one isolate was grouped with 2005 and 2010 cases of broilers with uneven growth (Group 3).

Phylogenetic analysis of FAdV isolates. Selected bursal homogenates were tested from the known FAdV-positive liver extracts. Regardless of the IBDV presence, all the FAdV sequences from the bursa were of FAdV type E (8b). These isolates shared 80 to 100% nt identity with the isolates from the liver as well as with the MK937074 FAdV reference sequence (data not shown).

Phylogenetic analysis of parvovirus NS gene sequences. PCR amplicons using primer sets PVF1 and PVR1 from selected bursal isolates were sequenced. Phylogenetic analysis revealed 94 to 98% overall nucleotide sequence identity in the NS region amongst bursal isolates as well as with the two reference strains (MN782009, JX114931, data not shown).

DISCUSSION

In this report, we have investigated the virus etiology of several atrophic bursal tissues suspected for IBDV. More than 50% of the tissues were negative for IBDV. All the IBDV sequences were grouped into the Genogroup 2 viruses which are predominantly variant viruses occurring in North America (11). Mutations in the right area, resorting of genome segments, and recombination can create an antigen change that is significant. It is important to continue monitoring IBD viruses from broilers and make sure current vaccination programs in breeders and broilers protect against broiler losses in productivity caused by field IBDV.

Avian reoviruses were readily isolated from bursal homogenates of young chicks from 29 out of 46 IBDV-negative tissues. This is consistent with the earlier reports showing the atrophy of lymphoid tissues following infection with virulent ARV (12). Recently, Egana-Labrin et al (13) have shown that virulent ARVs from the same genogroups

are responsible for inducing a variety of clinical conditions like malabsorption syndrome, uneven growth, viral arthritis, as well immunosuppression. For the past several years increased ARV activity has been reported in Georgia and elsewhere in the US and Canada (14). Several ARV variants associated with RSS cases were initially reported in 2006 (9) and subsequently from tenosynovitis and viral arthritis cases in Georgia (8, 9). Regardless of the geographic origins, these variants were grouped into at least four genogroups. Bursal isolates of ARV from the present investigation were closely associated with these genogroups sharing 85 to 98% nucleotide identity. These emerging variants seem to be antigenically distant from earlier cases of VA, but not distant enough to form a new cluster. These variants show a high percentage divergence from the established vaccine strains like S1133. This has an implication for the selection of autogenous vaccine candidates from representative genogroups of these variants. Protection of young chicks is achieved by vaccination of breeder stocks with the goal to prevent vertical transmission and to facilitate the passive transfer of immunity to young chicks. Many flocks are hyperimmunized with 3-5 shots of such reovirus vaccine combinations from local isolates with a live prime vaccine that exists, mostly 1133.

Virulent strains of FAdV cause outbreaks of IBH with or without the presence of IBDV or ARV with marked lymphoid depletion. This tropism for lymphocytes compromises both, humoral and cellular immune responses and increases the susceptibility to bacterial and opportunistic infections (1). All the sequences showed 81 to 99% nt identity to FAdV type E/8b. This is the most prevalent subtype in Georgia. Some integrators use inactivated autogenous vaccines to vaccinate breeders against FAdV. The goal is to prevent the vertical transmission of viruses implicated in IBH from the breeders to the broilers. The autogenous vaccines are mostly made from genotypes 8b and 11.

The role of parvoviruses as a primary pathogen is not well-established (15). Chicken parvoviruses have been implicated in RSS in broilers (16), and in pancreatic and bursal atrophy (1), and malabsorption syndrome (17). Aviparvoviruses are difficult to propagate in vitro. Currently, there are no commercial vaccines available. As with ARV, immunization of parents may offer maternal immunity to young chicks during the first few weeks of life but that is not included in commercial vaccine programs.

In conclusion, we were able to demonstrate the ARV, FAdV, and PV cause of bursal atrophy. These infections occur with or without the presence of infection with IBDV. These results provide some insights into which viral agents, other than IBDV, clinicians should consider when looking at bursal atrophy cases.

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Figure 1. Pie chart showing virus isolation/detection profile from IBDV-positive (A) and IBDV-negative (B) bursal tissues. IBDV-infectious bursal disease virus, FAdV- fowl adenovirus, ARV- avian reovirus, PV-avian parvovirus. N = a number of samples, "+" indicates mixed detection of the indicated virus group.

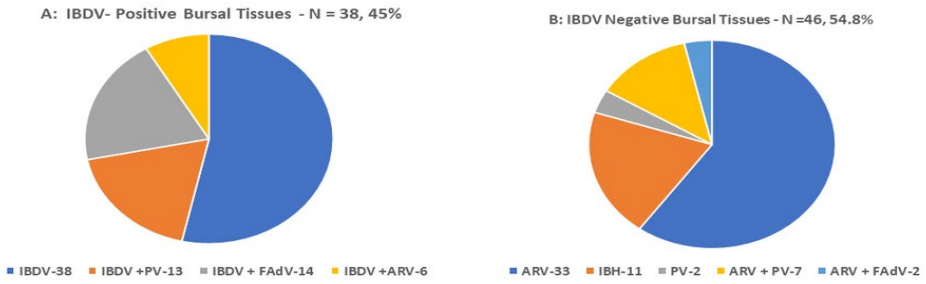


Figure 2. Percent nucleotide identity values for the IBDV-VP2 sequences of 490 nt of the Rt-PCR amplicon from bursal tissues were aligned to construct a phylogenetic tree using the neighbor-joining method. The analysis was conducted using MegAlign v15. GenBank accession numbers for reference strains GA198 variant, DelE variant, and STC classic strains are AY780340, D10065, and D00499, respectively.

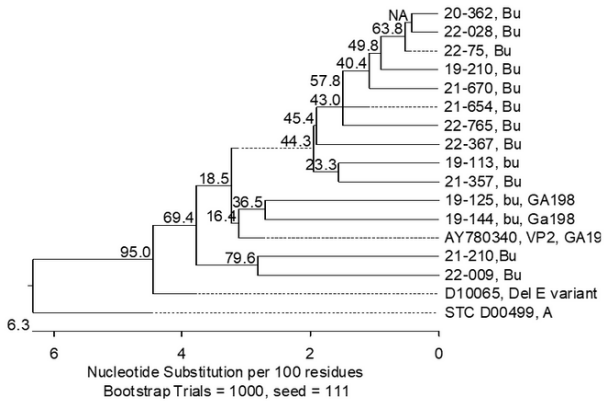
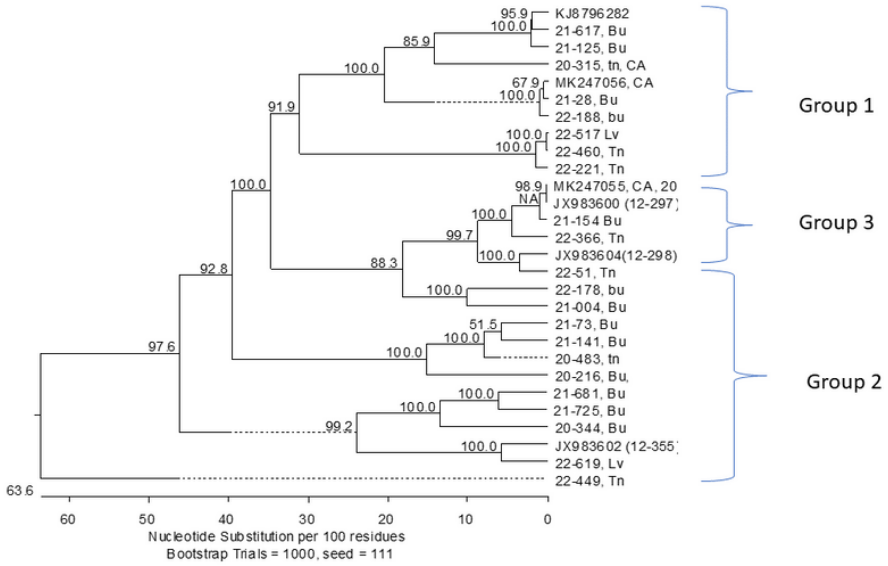


Figure 3. ARV-S1 nucleotide sequences of 900 nt of the Rt-PCR from bursal tissues were aligned to construct a phylogenetic tree using the neighbor-joining method (7). The analysis was conducted using MegAlign v15. Representative reference sequences from GenBank for each group were included in the analysis. These are: Gr 1 (KJ8796282, MK247056), Gr. 2 (JX983602), and Gr 3 (MK247055, JX983600, JX983604).



CONDUCTING A RISK ASSESSMENT DURING A HIGHLY PATHOGENIC AVIAN INFLUENZA OUTBREAK

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SUMMARY

Before the 2022 highly pathogenic avian influenza (HPAI) outbreak, the University of Minnesota Secure Food Systems (UMN SFS) team started work with a workgroup comprised of representatives from the turkey (commercial and breeder), broiler breeder, and egg layer sectors as well as state and federal regulators to create proactive risk assessments for high-risk bird transfer movements during a HPAI outbreak. When the 2022 HPAI outbreak began, the focus shifted from planning and preparing to outbreak response. With assistance from the workgroup, the team wrote and distributed four emergency-use permitted movement guidance documents to aid states and industry in the outbreak. The team obtained real-time data for guidance refinement for continued use. Although a full risk assessment is still in progress, proactive preparations allowed for timely drafting and vetting of these permitted movement guidance documents. Having resources to support business continuity for the poultry industry, particularly in the form of risk-based permitted movements, is an essential part of outbreak response, and proactivity is the best way to create those resources.

INTRODUCTION

The UMN SFS team conducts proactive risk assessments for animal and animal product movements originating from inside of a control area or infected state to a site within or outside of a control area to support continuity of business for agricultural industries. These risk assessments scientifically evaluate practices and procedures that are standard within an industry in the context of pathogen survivability and spread. Then, using both qualitative analyses and quantitative statistical and mathematical modeling, compare mitigation measures to reduce disease transmission within and between premises.

Following an adapted World Organization for Animal Health risk analysis approach (1), these assessments determine risk ratings for specific virus entrance and exposure pathways, as well as the overall risk of the specified animal or product movement. These ratings are determined based on the analytical work of the UMN SFS team in consultation with a collaborative workgroup comprised of animal industry representatives, state and federal agency representatives, and academicians. Risk assessments are then translated into permit guidance documents that serve as references and guidance for state and federal agencies when permitting movements during a foreign animal disease (FAD) outbreak. Use of SFS permit guidance documents is voluntary and varies state-to-state.

The UMN SFS team published their first risk assessments and permit guidances in 2009, starting with the lowest risk product movements (e.g., washed and sanitized egg movements). Since then, the team has 19 risk assessments and 31 permitted movement guidance documents that have been cleared by the United States Department of Agriculture Center for Epidemiology and Animal Health (USDA CEAH). As the industry becomes more familiar with risk assessments and permitted movement guidance documents, the team has been requested to evaluate the risk of animal and product movements perceived to be of higher risk, such as the movement of Raised-for-Release Mature Upland Game Birds to a Hunting Preserve or of live birds to off-site premises for continued production.

The process for writing risk assessments and permit guidance documents is intensive and requires a large time commitment, which is why the team attempts to write proactively; however, in some cases, risk assessments for needed/desired movements have been in progress when a disease outbreak occurs. At the onset of the 2022 HPAI outbreak, the risk assessment workgroup was in the process of writing four separate risk assessments for the movements of live birds to continued production (i.e., bird transfer movements): broiler breeder pullets to the breeder premises, layer pullets to the lay premises, commercial turkey poults to the grow-out premises, and breeder turkey hens to the lay premises. When a risk assessment for a movement is not yet completed or is in progress, an *ad-hoc* assessment of risk is often conducted by state and federal agencies for rapid decision-making. In 2022, to help incident command evaluate the risk involved in bird transfer movements, the UMN SFS team changed focus to create

emergency-use permit guidance documents for immediate use by the industry and state and federal officials overseeing permitting.

MATERIALS AND METHODS

Prior to the onset of the 2022 HPAI outbreak, the UMN SFS team had formed and regularly met with a collaborative, cross-commodity workgroup (CCWG), which was comprised of four distinct workgroups for the broiler breeder, egg-layer, commercial turkey, and breeder turkey industries. Each workgroup had the same 16 UMN SFS team members, 6 state representatives, 3 federal representatives, and 1 academician invited to the meetings with 7 broiler breeder representatives, 9 layer representatives, and 9 turkey representatives. The meetings were held virtually via the video conferencing platform, Zoom Video Communications, Inc. (San Jose, CA). The workgroups had 45 meetings with an average attendance rate of 70%, typically meeting every other week from January 2021 until November 2021, and resuming meetings in January 2022.

Discussions during workgroup meetings focused on pathways of virus entry into a flock and subsequent spread from the premises. Virus entry pathways are defined as: local area spread by: insects, aerosol transmission, wild birds (aquatic and non-aquatic), proximity to live-haul routes via feather, feces, and other fomites, movements of people, vehicles, or equipment (critical operational visits, people and their vehicles entering the premises, machinery or equipment shared between multiple premises, dead bird disposal, and garbage management), load-out operations, transport operations, and placement operations. Virus spread pathways were evaluated by determining the likelihood of detection when exposure happens prior to and during load-out. Entry pathway and spread analyses were based on a combination of standard industry practices and enhanced, outbreak-specific mitigations. In addition to the workgroup input, in 2021 an industry-wide survey was distributed, with the help of industry associations, via the online software Qualtrics (Provo, UT) to broiler breeder, egg layer, and commercial and breeder turkey veterinarians to capture industry movement practices.

In early February 2022, the first case of HPAI in the U.S. on a commercial turkey farm was reported. This and subsequent diagnoses were impediments to workgroup progress as veterinarians, academicians, and state and federal representatives were busy addressing the new HPAI outbreak. In order to accommodate the schedules of the workgroup, 14 meetings were held on an as-needed basis from February 2022 until September 2022. During meetings at the beginning of the 2022 outbreak, the UMN SFS team and the industry, state, and federal representatives worked together in one CCWG to discuss feasible mitigations that could be implemented industry-wide during an outbreak. Although a full risk assessment was still in progress, this information was then put into temporary, emergency-use permit guidance documents for immediate use.

UMN SFS permit guidance documents are harmonized across species and animal and product movements so that the movement-appropriate components are the same for the permit guidance documents, but the measures for the specific movements are unique to each species, animal, and product. The components for the permit guidance documents for bird transfer movements are defined in the following sections [1] requirements for a permit, [2] the Pre-Movement Isolation Period (PMIP), [3] product-specific biosecurity measures, [4] load-out biosecurity measures, [5] transport biosecurity measures, [6] placement biosecurity measures (including a post-placement isolation plan), and [7] active surveillance protocols. Requests for normal and outbreak-specific mortality data to assist in determining a PMIP duration and surveillance protocols were sent to the workgroup by the UMN SFS modeling team. Participation in the data requests was voluntary, and both the data received and participants were deidentified to maintain confidentiality and stored in a secured data cloud (Box, Inc., Redwood City, CA).

A discrete individual-based disease transmission model was used to simulate the HPAI transmission dynamics within a barn (2). The transmission model predicts the number of infectious and dead birds on various days post-flock exposure. An active surveillance simulation model was then utilized to predict the probability that an HPAI-positive bird would be sampled and detected via testing while considering the transmission model output, flock normal mortality (non-HPAI related), and the diagnostic sensitivity of the RRT-PCR test. The model input parameters for the disease state durations were estimated via experimental challenge study data provided by SEPRL (A/American Widgeon/SC/22-000345-001/2022 (H5N1) HPAIV). The adequate contact rate estimates, which determine the rate of HPAI spread in the disease transmission model, were based on analysis of outbreak mortality and diagnostic testing data using computational approaches. The poultry industry representatives in the CCWG provided the normal mortality data from uninfected flocks. Table A shows the PMIP duration and tentative surveillance schedules with the probability of detection for each commodity. As additional information became available, supplemental workgroup meetings and revisions occurred when schedules allowed.

RESULTS

By combining workgroup input, survey responses, and preliminary modeling results, four temporary, emergency-use permit guidance documents were prepared: movement of turkey hens from a single-age condition premises to a single-age breeder premises, movement of brooded poults from a single-age brood premises to a single-age grow-out premises, movement of broiler breeder pullets from a single-age pullet premises to a single-age breeder premises, and movement of a layer pullet premises to a lay premises. These guidance documents were shared with the industries and regulatory agencies by direct email communications and were uploaded to the Secure Poultry Supply (SPS) website (3). The permit guidance documents were used during the 2022 outbreak by regulatory officials in states that make use of SPS plans.

The industry-wide surveys and commodity-specific workgroups confirmed the most common movement type for each commodity, single-age to single-age for broiler breeders, and commercial and breeder turkeys, and multi-age to multi-age for layers, in order to focus efforts on assessing risk and mitigations for the more frequently requested movements. The permit guidance documents for single-age to single-age movements provided stipulations and caveats for movements that involve multi-age premises, as the team was unable to address these movement types in the permit guidance documents at the time. Industry producers and regulatory officials were advised to contact the UMN SFS team to discuss additional guidance for the layer or other multi-age movements. The risk ratings on the emergency-use permit guidance documents have a large range to account for the current uncertainty while the science is being evaluated. Any deviation from what is written in the permit guidance documents creates additional uncertainty. While the permit guidance documents are movement and sector-specific, certain components of the biosecurity, load-out, transportation, and placement sections contain common mitigations. The cross-commodity approach allowed for the sharing of individual commodity practices that benefited other sectors. Varying mitigations, such as the use of designated inside and outside load-out personnel, often started out as commodity-specific, but after discussions with all workgroups, they were decided to be feasible across commodities, and the mitigation was adopted into all permit guidance documents.

Preliminary modeling results defined tentative mortality triggers and surveillance schedules with at least a 95% probability of HPAI detection for both pre- and post-movement. As the outbreak continued, feedback on the permit guidance documents from one commodity was received, and additional modeling was completed for post-movement surveillance. Additional modeling for this and all other commodities was done and has shown that a probability of HPAI detection of at least 95% could be achieved with less testing than originally suggested, saving the industry and diagnostic laboratories time and resources. The workgroup discussed the new modeling results and adjusted the post-movement surveillance based on having at least a 95% probability of HPAI detection. However, not all protocols requested to be analyzed were able to achieve the desired probability of detection. One such was testing only day 4 post-movement to see if early detection could be achieved, but this protocol did not meet the desired probability of detection. Table A shows the probability of detection for each commodity for testing on day 4 post-movement only.

DISCUSSION

Work on completing the risk assessments for bird transfer movements is ongoing, as the UMN SFS team continues to perform qualitative and quantitative analyses. The current permit guidance documents are preliminary with tentative risk ratings, as final risk ratings from systematic risk analysis and modeling are unavailable at this time. The current risk ratings demonstrate the need for complete risk assessments, as the ratings have a wide range reflecting the uncertainty in the risk associated with the movements. The risk can be more closely determined by analyzing on a case-by-case basis but cannot be more refined until the full analysis is complete. Performing additional case-by-case analyses creates more responsibilities for all involved, which is yet another benefit of proactive analysis. The initial information obtained from the workgroup prior to the 2022 HPAI outbreak was intended for proactive use, however, the risk assessments and permit guidance documents were unable to be completed prior to the start of the 2022 HPAI outbreak when industry and regulatory officials sought bird transfer movement guidance. Had this preliminary work not been completed ahead of time, the creation of the temporary permit guidance documents would have taken longer, and more valuable time from industry and regulatory workgroup members would have been needed during the outbreak. The poultry industry and state and federal agencies have many duties to focus on during an outbreak and requesting their time away from these duties creates strain and additional stress. By having risk assessments and permit guidance documents completed proactively, industry and regulatory personnel can focus their time on outbreak response, and not actively work on new tasks. Additionally, having the risk assessments and permit guidance documents completed before the outbreak can assist with immediate outbreak response by having outlined guidance for movements. Although these documents are only used on a voluntary basis, they provide guidance to the industry

and regulatory officials and may assist in reducing the likelihood of HPAI virus spread, which is valuable for the management of an outbreak. While not always possible, proactivity is key in the development of tools for the poultry industry and regulatory industries to use at the onset of a disease outbreak, reducing the resources and time needed, preventing the spread of infection, and helping industry maintain continuity of business.

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Table A. Table A shows the PMIP duration, pre-movement surveillance, and the post-movement surveillance with the probability of detection for each permit guidance.

	PMIP Duration	Pre-movement Surveillance	Post-movement Surveillance	Predicted Detection Probability for Post-movement Surveillance	Predicted Detection Probability for testing on day 4 post-movement
Breeder Turkey Hens to the Lay Premises	8 days	Daily testing of all dead birds in all flocks in 11-swab pools (up to a maximum of 4 pools per flock/barn per day) by NAHLN labs beginning 8 days prior to loadout.	Testing of all dead birds in all flocks/barns in 11-swab pools (up to a maximum of 4 pools per flock/barn per day) by NAHLN labs on days 7 and 8 after placement.	0.95 (slow-spread scenario)	0.35 (slow-spread scenario)
Commercial Turkey Poults to the Grow-Out Premises	8 days	Daily testing of all dead birds in all flocks in 11-swab pools (up to a maximum of 4 pools per flock/barn per day) by NAHLN labs beginning 8 days prior to loadout.	Testing of all dead birds in all flocks/barns in 11-swab pools (up to a maximum of 4 pools per flock/barn per day) by NAHLN labs on days 7 and 8 after placement.	0.95 (slow-spread scenario)	0.35 (slow-spread scenario)
Broiler Breeder Pullets to the Breeder Premises	7 days	Daily testing of all dead birds in all flocks in 11-swab pools (up to a maximum of 4 pools per flock/barn per day) by NAHLN labs beginning 7 days prior to loadout.	Testing of all dead birds in all flocks/barns in 11-swab pools (up to a maximum of 4 pools per flock/barn per day) by NAHLN labs on days 6 and 7 after placement.	0.99 (baseline scenario)	0.48 (baseline scenario)
Layer Pullets to the Lay Premises	8 days	Daily testing of all dead birds, in flocks greater than 28 days of age, in 11-swab pools (up to a maximum of 4 pools per flock/barn per day) by NAHLN labs beginning 8 days prior to load-out.	Testing of all dead birds in all flocks/barns in 11-swab pools (up to a maximum of 4 pools per flock/barn) by NAHLN labs for days 7 and 8 after placement.	0.99 (baseline scenario)	0.60 (baseline scenario)

INFECTIOUS BRONCHITIS AND NEXT GENERATION SEQUENCING: THE BASICS AND PRACTICAL APPLICATIONS

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SUMMARY

Currently the reverse transcription quantitative polymerase chain reaction (RT-qPCR) test is a key tool for infectious bronchitis Virus (IBV) vaccines scientific and technical support. We use the RT-qPCR test for vaccine detection, passive surveillance, and active surveillance. There are limitations to the IB type specific RT-qPCR assays as changes to the short RNA sequence targeted by the RT-qPCR primers could result in a negative result. The purpose of this presentation is to introduce practical aspects of the implementation of Next Generation Sequencing (NGS) for the detection and identification of the majority of IBV genotypes and their variants in one test. IBV NGS technology improves upon the RT-qPCR assays and allows epidemiological analysis to quickly identify new and emerging IBV types associated with disease in the field.

INTRODUCTION

Infectious bronchitis (IB) is a highly contagious upper-respiratory viral disease of chickens caused IBV, an Avian Coronavirus. It has worldwide distribution and multiple antigenic types have been identified. New IBV types continuously arise from the accumulation of mutations in the spike (S1) protein.

The RT-qPCR is a highly specific and sensitive assay that can be used to detect IBV specific RNA and to detect IBV type specific RNA. The 5'UTR assay detects all IBV types while type specific assays target unique S1 sequences for GA08, vGA08, DMV1639, Ark, Mass, Conn, GA13 and Del072/GA98. Changes to the RNA sequence targeted by the RT-qPCR assay could result in a negative test even though the genotype is found in the sample. This is occasionally observed when the 5'UTR assay is positive, and the type-specific assays are negative. Traditionally, to determine the sequence and identify this IBV, the virus had to be isolated, purified, and then sequenced. The entire process is very time and labor intensive.

Traditional DNA sequencing began in the late 1960s/early 1970s. It took weeks or months to decode just a few bases of DNA. A leap forward was provided by Sanger et al in 1976 when Sanger sequencing was invented. With this method, several hundred bases of DNA sequence could be decoded in a day. Once paired with automation and other improvements, Sanger sequencing became the gold standard. Automated Sanger sequencing machines became commercially available circa 1987 that could decode about one thousand bases/day.

Today, NGS can be used to sequence thousands of bases a day without requiring the isolation and purification of the virus. Using Oxford Nanopore NGS technology, we have developed an IBV S1 sequencing workflow that allows for the detection of multiple genotypes present in one sample. This method bypasses the requirement of virus isolation resulting in faster turnaround times to not only identify current genotypes, but new variants arising in the field. In addition, veterinarians have access to needed epidemiological information to allow better decision making.

MATERIALS AND METHODS

The typical infectious bronchitis S1 NGS workflow includes sample preparation, RNA extraction, 5'UTR RT-qPCR to identify positive samples, followed by S1 sequencing and sequence analysis. The typical samples are tissue (trachea, cecal tonsils) or swabs (choana, cloacal). A combination of standard and proprietary methods is used for IBV S1 NGS.

RESULTS

Results from a broiler complex experiencing increased airsacculitis and mortality from E. coli polyserositis will be used to demonstrate the value of IB surveillance followed by S1 NGS. Results from IB surveillance via RT-qPCR showed increase detections of IB DMV1639. Most of the issues and DMV1639 detections were in a high-density broiler production area shared by multiple companies with differing IB vaccination programs.

As an example, RT-qPCR on a pooled trachea sample was 5'URT positive and positive for DMV1639 and Del072/GA98. The Del072/GA98 assay does not differentiate between Del072 and GA98. In contrast, NGS performed on the same pooled trachea sample detected both Del072 and GA98 in relative high abundance, DMV1639 in medium relative abundance and vGA08 in low relative abundance (not detected by RT-qPCR). It's important to mention that relative abundance is not a quantitative measurement but a description of read coverage for each genotype. Furthermore, NGS revealed the DMV1639 detected to be a vaccine strain used in neighboring complexes.

DISCUSSION

NGS expands our capabilities to provide scientific and technical support for IBV vaccines beyond the use of the RT-qPCR. Changes to the RNA sequence targeted by the RT-qPCR assay could result in a negative test even though the genotype is found in the sample. NGS results could be used to develop improved RT-qPCR assays based on new sequence data. NGS is an additional tool for veterinarians to make better decisions for the prevention and control of infectious bronchitis.

CASE REPORT ON THE RESPONSE TO VACCINATION OF TURKEY FLOCKS WITH HE VACCINE BY SPRAY

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INTRODUCTION

Hemorrhagic enteritis (HE) is caused by virulent strains of type 2 avian adenovirus. This virus can survive for extended periods of time in the environment because it is a non-enveloped virus. It is transmitted through oral or cloacal contact with infected feces. Disease is most seen in turkeys between six and eleven weeks of age (1). Turkeys under four weeks of age are generally protected by maternal antibodies. HE can cause clinical or subclinical disease. Clinical signs include bloody stool and depression (1). Mortality can range between 10 – 15% (1). Both clinical and subclinical infections cause immunosuppression so infected birds are prone to secondary bacterial infections (2). The HE virus targets both B cells and macrophages (3). Development of antimicrobial resistance and a subsequent effort to build programs with an emphasis on antimicrobial stewardship create the need to prevent immunosuppression so birds can fight bacterial infections without the use of antibiotics. Vaccination is important to control HE. The approved method of vaccination is via drinking water. Water disinfection needs to be turned off during water vaccination. Not all farms are designed to easily turn on and off disinfectants and some producers have concerns with birds being exposed to contaminated water once the disinfectant is off, leading to other disease challenges. The purpose of this case report was to investigate potential application of the HE vaccine via aerosol spray.

MATERIALS AND METHODS

Spray vaccination was trialed in 2022 on two turkey farms in British Columbia (BC) and one turkey farm in Alberta, Canada. Two flocks on each of the farms in BC and one flock on the farm in Alberta were spray vaccinated. The flock in Alberta contained 12,000 Nicholas toms. Farm A in BC placed 8,500 toms and Farm B placed 7,000 toms. The Alberta farm did not have a history of HE infection during the past year. This farm had previously given one HE vaccine via the water around 28 days of age. The producer was concerned about disease transmission from contaminated water when his water disinfected was off so requested to try spray vaccination. The same vaccine schedule was maintained, and a single dose was given via spray at 34 days of age. The two farms in BC historically had been vaccinated for HE but with recent concerns about the process and turning off the water sanitation system wanted to try spray application. A two-dose program via spray was implemented on both farms. The first flock trialed from farm A was vaccinated at 29 and 39 days while the second flock was at 27 and 37 days of age. Farm B vaccinated for both flocks at 28 and 42 days. All farms were vaccinated with a live avirulent Type 2 avian adenovirus (Oralvax HE® Merck Animal Health). Vaccination was applied with a coarse spray of a droplet size greater than 100 microns. Either distilled water or a vaccine stabilizer were used to mix the vaccine solution.

All farms collected 15 serum samples at the time of first vaccination to check maternal antibody status. Fifteen serum samples were then collected around one week after each vaccination to track maternal antibodies and vaccine response. Samples were collected on the Alberta farm one and two weeks after the single dose. A final 15 samples were taken in the period between three weeks post final vaccination and market depending on the farm. Exact timing is shown in Figure 1. Serum was only collected for Farm A flock 1 at first vaccination and 58 days of age. All titres were determined using ELISA. Histopathology was performed on spleens collected from cull birds on all farms one week after each vaccination. End of flock mortality was recorded for all flocks and health was monitored by the flock's veterinarian.

RESULTS

No flocks had maternal antibodies at the time of first vaccination. Flock 1 on Farm B was the only flock to show a low amount of seroconversion at one week post first vaccination (GMean = 141.94; %CV = 92.5%). At one week after the second vaccination Flock 1 on Farm B had only a small increase in titres (GMean = 283.58; %CV = 73.42).

Flock 2 at Farm A was the only other farm that showed seroconversion at this time (GMean = 2406.77; %CV = 71.38). All farms showed marked seroconversion by market age (Figure 1).

Histopathology on spleens from Farm A flock 1 at day 38 found one spleen with intranuclear inclusion bodies consistent with HE and two spleens that had no lesions. At day 52, this farm showed splenic hyperplasia with intralesional bacteria. Histopathology from Farm A flock 2 at day 35 found splenic hyperplasia with intravascular accumulations of bacteria. At day 50, this farm showed just splenic hyperplasia. Histopathology from Farm B flock 1 at both 36 and 50 days of age found splenic hyperplasia. Farm B flock 2 showed no lesions on histopathology for either sampling event. Histopathology from the Alberta farm found evidence of splenic hyperplasia with lymphoid depletion at both sampling events. Spleens taken at 41 days also found bacteria.

End of flock mortality for the AB farm was 8%. This was high for this barn; however, the flock experienced high morality in the first two weeks from septicemia. The morality attributed to the remainder of the flock life was only 4%, which is normal for this flock. Flock 1 and 2 from Farm A had 11.2% and 19.36% mortality respectively. Flock 2 had elevated mortality due to a pile-up and blackhead outbreak. The average mortality for this farm for 2021/2022 was 13.54%. The mortality for Farm B was 7.71% and 8.56% for flock 1 and 2 respectively. There was no clinical evidence of HE in any of the flocks as assessed by their veterinarians.

DISCUSSION

Since all farms achieved appropriate titer levels, this case report indicates that spray vaccination could potentially be an option for producers who have concerns with water vaccination. However, there are many areas that require further investigation. Further research should be done to determine when titers start to increase with spray. Research on experimental HE vaccines indicates that titers start to increase around four weeks post-vaccination (4). Maternal antibodies can be present up to six weeks of age (5). No farms had maternal antibodies remaining at four weeks of age, which indicates that all the farms had low maternal antibody protection. Therefore, it would not be expected to find antibodies on the samples taken within two weeks of the first vaccination, since this would be insufficient time for seroconversion to the vaccine and no maternal antibodies were remaining on these farms. However, there was early seroconversion in one flock at one-week post-vaccination and a second flock at two weeks post-vaccination. This may indicate an early possible field challenge in these flocks since they had low maternal antibody protection. However, histopathology did not find any lesions consistent with an HE infection on either of these farms.

Spray vaccination could also be a viable method of vaccination since flocks seroconverted in the absence of subclinical or clinical HE. There was one spleen in one flock that had intranuclear inclusion bodies that may indicate exposure to a field strain of HE. The splenic lesions seen later in this flock and in the other flocks were interpreted by the flock veterinarian as hyperplasia caused by bacterial septicemia. This diagnosis was based on the clinical picture seen on farm and the presence of bacteria on histopathology on Farm B and the Alberta farm. The mortality on the Alberta farm was not in excess of what is expected on that farm, which indicates that the change in vaccination technique did not have a negative impact.

Spray vaccination opens the option to vaccinate for HE to more farms regardless of their water disinfection system and allows farms with lower quality water or water contamination concerns to vaccinate while still protecting their birds. Vaccination of flocks struggling with HE challenges will reduce mortality in flocks that are clinical for HE and from secondary bacterial infections in both clinical and subclinical flocks. Improved immune function could reduce the amount of antibiotics needed in the industry and improve flock performance.

ACKNOWLEDGEMENTS

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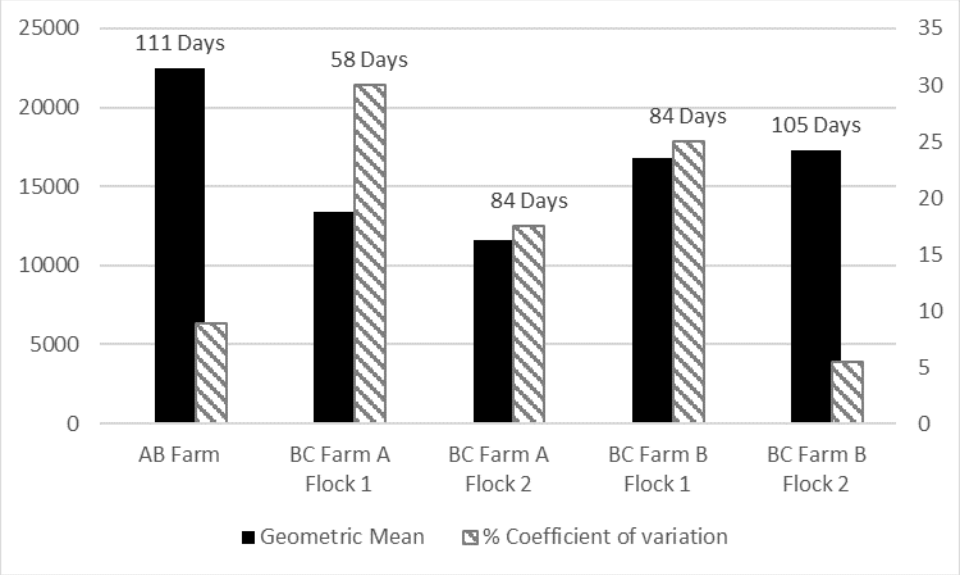
Figure 1. Geometric mean and percent coefficient of variation of the final titres for each flock with the age at which each set of titres was taken.



ENTEROCOCCUS CECORUM STRAINS ORIGINATING FROM LESIONS AND CLOACAL SWABS VARY IN THEIR RESISTANCE TO LYSOZYME

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SUMMARY

Enterococcus cecorum strains isolated from typical *E. cecorum* lesions in broilers cause more embryonic mortality after inoculation in albumen of embryonated eggs compared to strains isolated from the cloaca of healthy broiler reproduction chickens. Lysozyme, aside of the important role in the innate immune system, aids the antimicrobial capacity of the egg's albumen by inhibiting bacterial growth. Therefore, in the present study two experiments were performed to test the lysozyme resistance of 14 lesion strains and 14 cloaca strains. For all *E. cecorum* strains, the minimal inhibitory concentrations (MIC) of lysozyme were determined using an agar dilution method and the growth rates in broth with (500 and 2000 µg/mL) or without lysozyme were assessed. Thirteen cloacal strains had a MIC ranging from 1000-8000 µg/mL, while one cloacal strain and all lesion strains were resistant to the lysozyme concentrations tested. Growth rates of cloaca strains were significantly lower compared to lesion strains at both tested lysozyme concentrations. Lysozyme resistance of lesion strains could enhance survivability in hostile environments.

INTRODUCTION

Clinical outbreaks of *Enterococcus cecorum* are characterized by lameness or paralysis of affected birds and the typical macroscopic lesions are pericarditis, arthritis and osteomyelitis of the femur, tibia and the 6th thoracic vertebra (1). An outbreak can have a major economic impact as mortality and condemnations rates can be elevated. No specific *E. cecorum* strains are found to be responsible for outbreaks in chickens, but strains isolated from lesions (pathogenic strains) are genetically less diverse compared to strains isolated from the cloaca (commensal strains) (2, 3). This suggest that pathogenic *E. cecorum* strain have specific traits for virulence, identification of virulence traits could be used to differentiate pathogenic strains from commensal *E. cecorum* strains.

In different studies an embryo lethality assay was used to type *E. cecorum* isolates (4, 5). After inoculation of 12-day embryonated broiler eggs in the albumen, mortality rates of lesion strains were much higher compared to cloaca strains. This difference in mortality rates could be explained by the differences in virulence and sensitivity to the antimicrobial effects of albumen (5).

The albumen protects the embryo against microbial infection, a major antimicrobial component of the albumen is lysozyme. The antibacterial effects of lysozyme depends on the hydrolysis of cell wall peptidoglycan and on a cationic antimicrobial peptide activity causing membrane permeabilization, which lead to death of both gram-positive and gram-negative bacteria (6). In addition to its role in the albumen, lysozyme also plays a major role in the innate immune system. The protein is excreted in mucus in the oviduct, lung and intestines. Pathogenic gram-positive bacteria, e.g. *Enterococcus faecalis*, were shown to be resistant to lysozyme (7). The resistance of pathogenic or commensal *E. cecorum* strains to lysozyme is unknown.

The aim of this study was to examine the resistance to lysozyme of *E. cecorum* strains originating from the cloaca of healthy broiler reproduction chickens (cloaca strains) and strains originating from typical lesions in broilers (lesion strains). We hypothesized that lesion strains have a higher lysozyme resistance than lesions strains. To test this hypothesis two experiments two experiments were performed. 1) The minimal inhibitory concentrations of lysozyme were determined for 14 cloaca strains and 14 lesion strains using a agar dilution method. 2) Bacterial growth curves of the *E. cecorum* strains without and with 500 and 2000 µg/mL lysozyme were compared.

MATERIALS AND METHODS

***E. cecorum* strains.** The 28 *E. cecorum* strains used in this study were previously described by Manders et al., 2022 (5). Briefly, 14 *E. cecorum* strains were isolated from cloacal swabs taken from clinical healthy broiler

reproduction flocks (cloaca strains). Bacterial culture of swabs of typical *E. cecorum* lesions (pericarditis, femur head necrosis, arthritis and spondylitis) in broilers was performed and when *E. cecorum* was isolated in pure culture these strains were preserved (lesion strains). Commensal *E. cecorum* bacteria vary in their ability to metabolize mannitol while pathogenic strains are not able to metabolize mannitol (3, 8). Therefore, only *E. cecorum* strains originating from cloaca swabs which were able to metabolize mannitol and only *E. cecorum* strains isolated from lesion which were unable to metabolize mannitol were included. For both cloaca and lesion strains, only one *E. cecorum* strain per flock was selected. Whole genome sequencing showed that the strains were not clonally related (5).

Inocula preparation. *E. cecorum* strains were recovered from cryopreservation, plated on Columbia agar plates supplemented with 5% Sheep Blood (CBA) (BD, Franklin Lakes, USA) and after 24 hours incubation at 37°C in a 5% CO_2 enriched atmosphere colonies were scraped of the plate and suspended in physiological saline (PS) to an optical density of 0.5 McFarland (McFarland Densitometer, type DEN-1, Grant instruments Ltd., Shepreth, England), which corresponds with approximately 10^8 CFU/mL. This suspension was serially diluted with peptone physiological saline for inocula for experiment 1 and with Todd-Hewitt broth with 1 % yeast extract (THBY) for experiment 2 to a final concentration of approximately 10^6 CFU/mL. The final bacterial concentrations of the inocula were assessed by colony counting of serial dilutions on CBA.

Experiment 1 – Lysozyme MIC assay. Columbia blood agar plates with or without hen egg white lysozyme (62970-1G-F, Sigma-Aldrich, Zwijndrecht, the Netherlands) were made in-house. Two-fold increasing concentrations of lysozyme ranged from 62.5-8000 µg/mL. Minimal inhibitory concentrations (MICs) were determined by spotting a droplet of 10 µL inoculum on the CBA plates (approximately 10^4 CFU/spot). In total 21 CBA plates per lysozyme concentration were produced, per plate 4 different strains and a negative control (physiological saline) were spotted (7 different plates) and per strain and lysozyme concentration the MIC assay was performed in triplicate. Bacterial growth was evaluated after incubation at 37°C in a 5% CO_2 enriched atmosphere for 24 hours. The minimal concentration of lysozyme in which no bacterial growth was observed was designated as the MIC value. In case the MIC values differed in between the three replicates, the highest MIC value was given.

Experiment 2 - Bacterial growth assessment. The bacterial growth of all 28 *E. cecorum* strains in THBY and THBY with two different lysozyme concentration were assessed. Wells of a sterile 96-well flat bottom microplate were filled with 100 µl of TBHY with 10^6 CFU/mL suspended *E. cecorum* bacteria (inocula) and with 100 µL of TBHY without lysozyme or with a lysozyme solution of 1000 or 4000 µg/mL in TBHY (final lysozyme concentrations 500 and 2000 µg/mL, respectively).

Microplates were incubated at 37°C in a 5% CO_2 enriched atmosphere and the optical density (OD) at 600 nm was measured (Synergy HTX multi-mode microplate reader, BioTek® instruments, Winooski, Vermont, USA) every 10 minutes for 16 hours. In between measurements the microplate was shaken. Bacterial growth was assessed in duplicate per strain and per control/lysozyme concentration. For each strain the maximum OD was determined and the time to reach an OD of 0.2 was calculated using GraphPad Prism version 9.3.1 (9).

Statistics. The time to reach an OD of 0.2 and the maximum OD were compared using One-way ANOVA followed by Tukey HSD as a post hoc test (10). Differences were considered significant if $p < 0.05$.

RESULTS

Experiment 1 – Lysozyme MIC assay. In the agar dilution MIC assay all lesion strains (n=14) were resistant to the lysozyme concentrations tested (MIC > 8000 µg/mL). Thirteen cloacal strains had a MIC ranging from 1000-8000 µg/mL, while one cloacal strain was resistant (MIC > 8000 µg/mL). One cloaca strain had a MIC of 1000 µg/mL, seven cloaca strains had a MIC of 4000 µg/mL and five cloaca strains had a MIC of 8000 µg/mL. In all cases the MIC values were the same on the three different plates per lysozyme concentrations tested, except for one cloaca strain. For this strain no bacterial growth was observed on one plate with 4000 µg/mL while on the other two plates one and five colonies were present and the MIC was defined as 8000 µg/mL.

Experiment 2 - Bacterial growth assessment. The mean bacterial growth of the cloacal and lesion strains grown in THBY with or without the addition of lysozyme are shown in Figure 1.

The time to reach an OD of 0.2 was significantly longer for cloaca strains when lysozyme (500 and 2000 µg/mL) was added in the THBY when compared to the group with no lysozyme. A similar pattern was seen for the maximum OD, these were significantly lower in the groups with 500 and 2000 µg lysozyme/mL (Table 1).

The time to reach OD of 0.2 and the maximum OD differed significantly for the lesions strains when cultivated with 2000 µg lysozyme/mL compared to lesion strains cultivated without lysozyme or with 500 µg lysozyme/mL. No significant difference were observed for the time to reach OD of 0.2 between the lesion strains cultivated in 500 or 2000 µg lysozyme/mL (Table 1).

All lesion strains reached an OD of 0.2. Not all cloaca strains reached an OD of 0.2, the maximum OD of five cloaca strains at a lysozyme concentration of 500 and 2000 µg/mL ranged from 0.116-0.199 and 0.025-0.189, respectively. Only one strain did not reach OD of 0.2 in both concentrations.

DISCUSSION

In this study the resistance to lysozyme was tested for *E. cecorum* strains isolated from cloacas of healthy broiler reproduction chickens or from typical *E. cecorum* lesions. Based on a lysozyme MIC agar dilution assay and assessment of bacterial growth in THYB in the presence of lysozyme, we conclude that lesion strains are more resistant to lysozyme compared to cloaca strains. Lesion strains were resistant to 8000 µg/mL lysozyme in the MIC agar assay and only the highest lysozyme concentration tested (2000 µg/mL) had a significant effect on the growth rates of lesion strains. While for most cloaca strains (13/14) a MIC for lysozyme could be determined and 500 µg/mL lysozyme had a detrimental effect on bacterial growth (Figure 1 and Table 1).

A previous study by our group showed that virulence typing of different *E. cecorum* isolates can be done using an embryo lethality assay (ELA) where bacteria are inoculated in the albumen of 12 day incubated eggs (5). In this current study, the same set of *E. cecorum* strains were used. The results of the MIC assay corresponded with the results of the ELA, most cloaca strains (13/14) were not resistant to lysozyme and also did not cause or caused limited embryonic mortality in an ELA. For lesion strains, the opposite was true. These strains were resistant to lysozyme and caused high mortality rates in the ELA. The lysozyme MIC assay might be a replacement for the ELA as a quick virulence screening test for E. cecorum isolates. However, validation of the MIC assay with more E. cecorum strains is required.

Three different types of lysozyme have been identified, c-type (chicken type), g-type (goose type) and i-type (invertebrate type). The major lysozymes produced by mammals are of the c-type and are present in high concentrations in different body fluids (>500 µg/mL) (6, 7). In chickens, c-type lysozyme is predominantly found in the oviduct and the egg. In the intestines of young chickens up to 8-day old, the expression of c-type lysozyme was found, while in the intestines of older birds, as well as in lungs and in bone marrow, the g-type lysozyme gene was expressed (11, 12). In the present study hen egg white lysozyme was used, which consists of c-type lysozyme. It might be expected that *E. cecorum* strains are equally resistant to both lysozyme types as, c-type and g-type lysozymes both have strongly similar antigenic properties (11).

Higher tolerance to lysozyme (up to 50 mg/mL) have been found in pathogenic *E. faecalis* and *Staphylococcus aureus*, when compared to other gram-positive bacteria (7). Lysozyme resistance mechanisms in bacteria are based on either 1) modifying the peptidoglycan in order to protect against hydrolysis by lysozyme, 2) alternating bacterial envelope charge or 3) expressing lysozyme inhibitors (6). These mechanism have not been described for *E. cecorum* and would be of interest of further research to unveil potential differences in pathogenesis between cloaca and lesion strains.

In conclusion, we showed that *E. cecorum* strains isolated from lesions were more resistant to lysozyme compared to strains isolated from cloacas. A lysozyme MIC assay could be a quick screening test for virulence typing of *E. cecorum* isolates.

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Figure 1. The effect of lysozyme on the growth of cloaca and lesion associated *Enterococcus cecorum* strains. The effect of 0, 500 and 2000 µg/mL lysozyme on the growth of cloaca (n=14) and lesion (n=14) *Enterococcus cecorum* strains cultured in Todd-Hewitt broth with 1% yeast at 37° C in a 5% CO_2 enriched atmosphere was assessed by measuring the optical density (OD) at 600 nm.

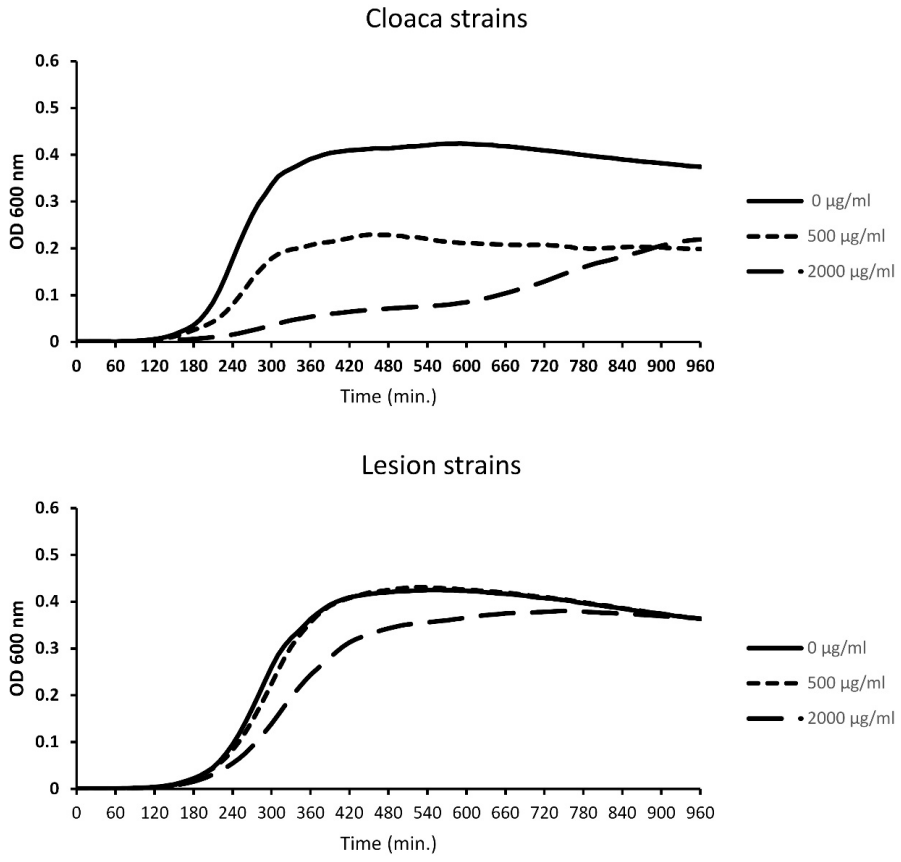

Table 1. The effect of 0, 500 and 2000 µg/mL lysozyme on the bacterial growth of 14 cloaca and 14 lesion associated *Enterococcus cecorum* strains.

	Cloaca strains			Lesion strains		
Lysozyme (µg/mL)	n=	Time (min.) to 0.2 OD (SEM)	Maximum OD (SEM)	n=	Time (min.) to 0.2 OD (SEM)	Maximum OD (SEM)
0	14	250 (7.0)	0.44 (0.02)	14	292 (12.9)^{C}	0.51 (0.01)^{E}
500	14[1]	486 (91.4)^{A}	0.26 (0.02)^{B}	14	309 (14.2)^{CD}	0.46 (0.02)^{E}
2000	14[1]	669 (86.1)^{A}	0.22 (0.03)^{B}	14	362 (24.9)^{D}	0.40 (0.02)

Bacteria were cultured in Todd-Hewitt broth with 1% yeast with 0, 500 and 2000 µg/mL lysozyme at 37°C in a 5% CO_2 enriched atmosphere and the optical density at 600 nm was measured every 10 minutes for 16 hours.
[1]Five of the fourteen cloaca strains did not reach an OD of 0.2 in the presence of 500 or 2000 µg lysozyme/mL.
^{ABCDE}Values within a column with the same superscript do not significantly differ ($p > 0.05$).

DEVELOPING A NOVEL METHOD FOR SEQUENCING THE WHOLE GENOME OF INFECTIOUS BURSAL DISEASE VIRUS FROM FIELD SAMPLES

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SUMMARY

Infectious bursal disease virus (IBDV) is a ubiquitous virus that causes immunosuppression and mortality in poultry1, 3. It is common practice in the poultry industry to submit samples (primarily bursal tissues) that originated in the field for viral isolation, detection, and genomic classification of IBDV using classical and PCR laboratory methods2. However, these methods can consume valuable time and resources. Thus, we sought to develop an accurate and cost-effective method for sequencing the whole genome, including Segment A of IBDV positive field samples. By combining novel lab protocols, nanopore-based sequencing, and custom bioinformatic tools, we can sequence, assemble, and analyze genome Segments A directly from field samples and autogenous vaccine. This information can be used to genotype IBDV that is present in field samples and culture. Analysis of this information can help stakeholders to make informed decisions as it pertains to protecting poultry flocks from IBD.

MATERIALS AND METHODS

Nucleic acid was extracted from bursal tissue samples sourced from various broiler farms. Bursal tissue was prepared by manual homogenization in phosphate buffered saline (PBS). The Zymo Research ZymoBIOMICS DNA/RNA MiniPrep kit was used to purify and extract viral RNA. After purification, IBDV RNA was quantified using the real-time RT-qPCR assay described by Techera, et al. (Table A).

Reverse transcription was performed in a one-step reaction using Invitrogen SuperScript IV Reverse Transcriptase and either random or specific primers. Sequencing libraries were prepared using the PCR-cDNA Barcoding kits from Oxford Nanopore Technologies (ONT), and multiplexing was available for up to 24 samples. The amplified cDNA libraries were sequenced using an ONT flow cell (version R9.4.1) and a GridION Mk1 or MinION Mk1B.

Sequence data was analyzed using proprietary software and workflows developed by Ceva Animal Health; this was accomplished by using Geneious Prime (version 2022.0.2) bioinformatics software.

RESULTS

IBDV-specific RT-qPCR resulted in detectable viral loads in tissue samples, isolation of the full Segment A, and accurate typing of all IBDV was accomplished using proprietary software and workflow within Geneious Prime. Typing was confirmed by comparison to short-read sequence data performed by a separate internal laboratory.

CONCLUSIONS

This cost-effective process of RNA extraction, library preparation, sequencing, and data analysis allows for accurate typing of IBDV within 48 hours. Additionally, the entire Segment A genome was sequenced. Rapid sequencing and typing can improve the sample turnaround time in both research and diagnostic laboratories, aiding in vaccine development and disease prevention.

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Table 1.

Assay	Oligo name	Oligo sequence (5’ -> 3’)
Techera IBDV RNA	F143	CMAGATCAAACCCAACAGATTGT
	R253	CTCTGACCTGAGAGTGTGCTTCTC
	S175	ACGGAGCCTTCTGATGCCAACAACC

ANTIMICROBIAL USE AND PHENOTYPIC ANTIMICROBIAL RESISTANCE IN CALIFORNIA BACKYARD POULTRY

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SUMMARY

As backyard poultry (BYP) ownership increases across California, information on bacterial antimicrobial resistance (AMR) levels across this community remains sparse. To understand how resistance levels among bacteria associated with BYP relate to husbandry practices and how these patterns of AMR compare to observed levels in the commercial poultry production, this study examines samples collected over four seasons from 36 BYP premises across California. Analysis of phenotypic resistance finds the resistance patterns resemble those from commercial poultry-associated bacteria surveilled the National Antimicrobial Resistance Monitoring System (NARMS), with most AMR levels being comparable to or lower than those in the NARMS data set. Additionally, multilevel logistic regression found no significant differences between premises that had used antimicrobials within the last year and premises that had not used antimicrobials.

INTRODUCTION

The last two decades have seen a rapid ascent in the popularity of BYP ownership among California residents (1). As the number of BYP owners in the state has surged, systems for providing information on husbandry, antimicrobial stewardship, and biosecurity have struggled to keep up (2, 3). In the face of this challenge, the importance of understanding the threat of antimicrobial resistance (AMR) facing this community has grown. Whereas AMR among commercial poultry is monitored by the NARMS, no such surveillance is established to track resistance among BYP. Little is known about resistance levels among bacteria associated with BYP in California and the effects of husbandry practices and other factors on those levels. As a result, this community has the capacity to serve as small reservoirs of AMR genes, given the variability in husbandry practices and the lack of regulation for hobbyists. This longitudinal study examines the resistance levels of BYP premises across California to gain some insight into current patterns of resistance among this population and how they relate to those in commercial poultry.

MATERIALS AND METHODS

To survey resistance levels of BYP premises across the state of California, a longitudinal survey of 36 premises selected from a Google Form-based survey was conducted, with 16 premises that used antimicrobials within the last year and 20 that had never used antimicrobials were selected from 162 survey respondents who lived in California. The sixteen antimicrobial-using (AU) premises consisted of all respondents who fit the criteria of having used antimicrobials within the previous year, lived in California, and other criteria specified in the study design. The antimicrobial-free (AF) premises were selected to be geographically matched pairs with the AU premises, along with 4 additional "buffer" premises included in case of non-compliance by participants. Under the guidance of the Pitesky lab, participants collected 3 cloacal swabs and 1 environmental boot swab at corresponding times for four consecutive seasons: Summer 2018 (Q1), Fall 2018 (Q2), Winter 2019 (Q3), and Spring 2019 (Q4). These samples were then mailed to the Pitesky Lab (shipments were kept cold throughout this process) for selective culturing and isolation. The four bacteria targeted for isolation were: *Enterococcus* spp., *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp. Isolates were sequenced by the California Department of Public Health and tested for phenotypic resistance using four antimicrobial susceptibility testing (AST) panels for the corresponding bacteria to obtain minimum inhibitory concentrations (MICs). *E. coli* and *Salmonella* spp. isolates were tested against the NARMS gram negative panel and an avian panel, Enterococcus isolates were tested against a NARMS gram positive panel, and a *Campylobacter*-specific panel was used to assess resistance among the *Campylobacter* isolates. Findings from the gram-positive panel, campylobacter panel, and gram-negative panel were assessed using NARMS breakpoints, which are established with regards to treating infections in humans, rather than poultry. In following with the practices of NARMS Integrated Reports, intermediate (I) resistance isolates were grouped with the susceptible (S) isolates when calculating percentage of resistant (R) isolates. Resistance levels were compared to the NARMS 2019 Integrated Report (4).

RESULTS

In total, for both types of swabs across all four seasons, 238 *Enterococcus* spp. (100% isolation), 247 *Escherichia coli*, 13 *Campylobacter* spp. (5.88% isolation), and 5 *Salmonella* spp. (3.78% isolation) isolates were recovered. 212 of the 238 samples collected yielded *E. coli* (89.08% isolation), but 247 unique isolates were collected because multiple isolates were collected when multiple morphologies were present during culturing for isolation.

Among the five *Salmonella* isolates, no phenotypic resistance was found for any of the tested antimicrobials on the gram-negative panel (there are no NARMS breakpoints for the avian panel). Among the *E. coli*, the overall resistance levels among the BYP isolates were typically similar to or below those observed in NARMS's three classes of isolates from poultry: Retail Chickens, Chickens (Product), and Chickens (Cecal). Among the *E. coli* isolates, resistance was most prevalent for tetracycline and streptomycin (see Table 1), though resistance to those antimicrobials was less common among the BYP isolates than the NARMS poultry data. Among the gram positive bacteria (*Enterococcus*), only ciprofloxacin resistance among *Enterococcus* faecium was notably more prevalent for the BYP isolates than in the NARMS poultry data. Other resistance levels were comparable to or lower than the NARMS findings. Among Campylobacter, the general patterns of resistance among NARMS poultry isolates were also observed in the 13 isolates from the BYP premises with some minor differences in tetracycline and quinolone resistance levels likely attributable to the small sample size.

Statistical analysis of the categorized MIC data using multilevel logistic regression models to account for the sampling structure was performed to examine the differences in resistance levels due to antimicrobial use, adjusted for season, swab type, and species (when applicable) found no significant differences in resistance prevalence due to antimicrobial usage.

DISCUSSION

In general, the resistance patterns observed in the BYP isolates bore a striking resemblance to the patterns from the NARMS 2019 Integrated Report. These similarities could be attributed to any number of potential reasons, such as these patterns of AMR being typical of bacteria associated with poultry regardless of husbandry practices or changes in antimicrobial stewardship in response to guidance such as the Veterinary Feed Directive and California Senate Bill 27. While the analysis showed no significant differences in resistance levels between the AU and AF groups, it does merit noting that for many of the antimicrobials tested, resistance levels in both groups were very low, making it unlikely to find substantial differences in resistance patterns. Furthermore, the type of antimicrobial used and the frequency and dosage at which it was used within the prior year varied among the AU premises, making it more likely any effects would be diluted across the entirety of the sample. Further longitudinal research with more specific selection criteria or experimental studies could examine the effects of a single drug class on resulting resistance levels, potentially starting from day of administration.

ACKNOWLEDGEMENTS

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Table 1. Phenotypic resistance of *Escherichia coli* and *Salmonella* spp. BYP isolates to gram negative AST panel separated by whether the premise did (Abx) or did not (AF) use antimicrobials on their backyard chickens. Resistant isolates are defined here as falling into the Resistant category based on NARMS clinical breakpoints.

Phenotypic Resistance of Gram Negative Bacteria

Number of Resistant Isolates (I or R)

CLSI Class & Agent	Antimicrobial Rank	E.coli [Abx] (114)	Salmonella [Abx] (2)	E.coli [AF] (133)	Salmonella [AF] (3)
Beta Lactam Combination Agents					
Amoxicillin-clavulanic acid	CI	1 (0.9%)	0	2 (1.5%)	0
Penicillins					
Ampicillin	CI	6 (5.3%)	0	3 (2.3%)	0
Macrolides					
Azithromycin	CI	0	0	0	0
Phenicols					
Chloramphenicol	HI	0	0	1 (0.8%)	0
Quinolones					
Ciprofloxacin	CI	0	0	0	0
Nalidixic acid	CI	2 (1.8%)	0	1 (0.8%)	0
Cephems					
Ceftriaxone	CI	1 (0.9%)	0	2 (1.5%)	0
Cefoxitin	HI	1 (0.9%)	[†] 0	2 (1.5%)	[†] 0
Ceftiofur	CI	0	0	2 (1.5%)	0
Aminoglycosides					
Gentamicin	CI	2 (1.8%)	[†] 0	5 (3.8%)	[†] 0
Streptomycin	CI	14 (12.3%)	[†] 0	10 (7.5%)	[†] 0
Folate Pathway Antagonists					
Sulfisoxazole	HI	9 (7.9%)	0	5 (3.8%)	0
Trimethoprim-sulfamethoxazole	HI	2 (1.8%)	0	1 (0.8%)	0
Tetracyclines					
Tetracycline	HI	16 (14.0%)	0	31 (23.3%)	0

[†] CLSI advises that Salmonella spp isolates should not be reported as susceptible to aminoglycosides or 1st and 2nd generation cephalosporins, which may appear active in vitro against Salmonella spp but are not clinically effective.

EPIDEMIOLOGICAL PATTERNS OF THE INFECTIOUS CORYZA OUTBREAK IN CALIFORNIA 2019-2022

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SUMMARY

Infectious coryza (IC) is the disease caused by the bacterium Avibacterium Paragallinarum. IC is an important disease in the state of California due to increasing prevalence and economic impacts on the egg and meat producers in the state. IC has been shown to cause poor growth in broilers and decreased egg production in laying hens. Assessment of the epidemiologic characteristics of infectious coryza were performed to better understand the spread of the disease through poultry populations using data from the California Animal Health and Food Safety laboratory system (CAHFS). Factors that were assessed include: production type, season, spatial distribution, age, and co-infections. Production class was defined as broiler, layer, or backyard birds based on submitter's information.

MATERIAL AND METHODS

Cases used in this study were obtained from the CAHFS laboratory system. A case was defined as coryza suspicious individuals from which swabs were collected and analyzed by a polymerase-chain reaction (PCR) from January 2019 through May 2022. Samples were taken from the respiratory tract, specifically the choanal cleft from birds presented to the lab. The presence of co-infecting pathogens was determined using culture and PCR. Seasonality was analyzed by sorting data into winter (December-February), spring (March-May), summer (June-August), and fall (September-November). Spatial distribution was evaluated by county. A univariate logistic regression model was used to evaluate the association between individual potential predictors and Infectious coryza in poultry. Odds ratio (OR), confidence interval (CI), were reported and a p value < 0.05 considered statistically significant.

RESULTS AND DISCUSSION

A total 1278 cases were screened for coryza by PCR, from those, 461 were positive and 817 negatives. Affected production type was shown to be a statically significant predictor of IC risk. We found that backyard birds had 5.1 times more odds of having IC than broilers (95% CI 3.7-7.0, OR =5.1, p = 0.00). On the other hand, layers had 4.8 times more odds of having IC than broilers (95% CI 3.4-6.8, OR = 4.8, p =0.00)). This finding, increased level of positives within the backyard populations, suggests that backyard flocks are important reservoirs for IC (Table 1).

Regarding seasonality, winter was used as the reference value due to the assumption that respiratory diseases are often more prevalent during winter months. The only season that had a statistically significant predictive value was summer with an odds ratio of 1.6 (95% CI 1.1, 2.3, OR =1.6, p = 0.008) when compared to winter. Spring and fall did not have statistically significant predictive values. These results suggest a higher prevalence of IC in summer compared with other seasons of the year for the period analyzed.

Co-infecting pathogens were evaluated within the various chicken production types. Among broilers that tested positive for IC, the most common coinfections were infectious bursal disease virus (54.5%) followed by infectious bronchitis virus (48.5%), *E. coli* (33.3%), *Ornithobacterium rhinotrachealis* (9.1%), and some cases without any co-infecting pathogen (16.7%). In layers that tested positive to IC the most common coinfections were Mycoplasmas (30.7%), *E. coli* (30.7%), no co-infection (24.8%), infectious bronchitis virus (15.7%), and infectious laryngotracheitis virus (15.7). Overall, these pathogens are expected since are endemic to poultry populations and contribute to complex upper respiratory diseases i.e., IBV, ILT, ORT, and mycoplasma. On the other hand, IBDV might be reflecting an immunosuppressive component on this respiratory issue.

Spatial distribution was analyzed using the information on county submission. Counties with the most positive cases were Merced (51 layers, 47 broilers, 1 backyard), Stanislaus (34 layers, 11 broilers, 19 backyard), San Joaquin (25 layers, 1 broiler, 25 backyard), Los Angeles (1 layer, 0 broilers, 37 backyard), San Diego (9 layers, 1 broiler, 14 backyard), Sonoma (10 layers, 2 broilers, 8 backyard), San Bernadino (13 layers, 0 broilers, 7 backyard), and Riverside

(5 layers, 0 broilers, 14 backyard) (Figure 1). Overall, the significance of this data is difficult to interpret but reflect the high representation of backyard flocks on each of the studied county. Finally, age was not a statistically significant factor in IC epidemiology in this study.

CONCLUSION

The major take aways from this study was increased prevalence of IC in backyard birds and layers as well as increased prevalence during the summer months. Backyard birds are most likely an important reservoir for IC with important impact on the poultry industry in California. Since IC is quickly killed outside of the host, understanding possible reservoirs for the bacterium is important in understanding the epidemiology of the disease and these findings emphasize the importance of proper biosecurity to create a barrier between backyard birds and the commercial flocks, as well as between layer and broiler flocks.

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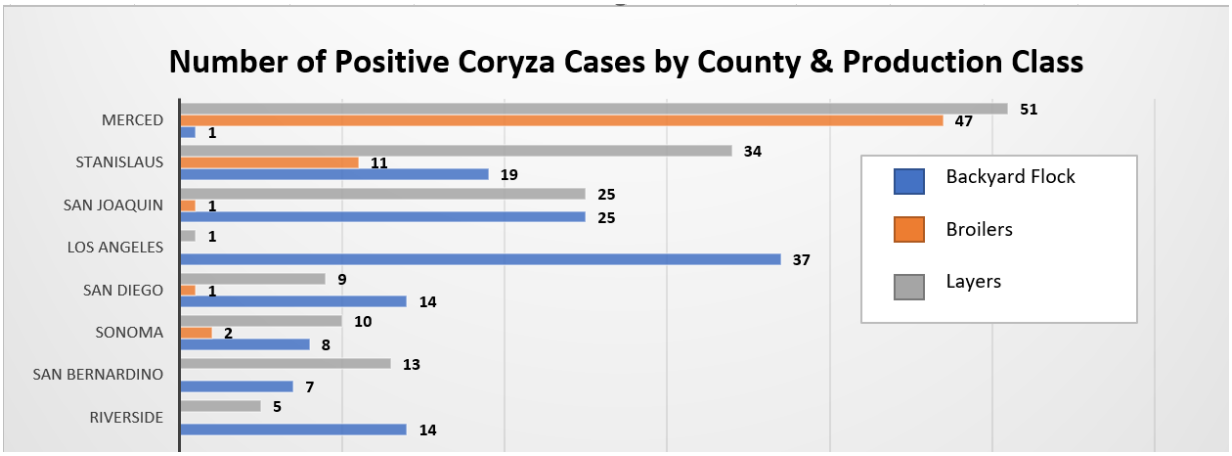
Table 1. Shows the odds ratios of having IC cases for each of the chicken production types investigated using Meat birds as a reference.

Variable	n	Odds Ratio	95% CI	SE	z value	p-value
Class						
Meat	438	ref	ref	ref	ref	ref
Layers	331	4.8	(3.4,6.8)	0.83	9.1	0.00
Backyard	509	5.1	(3.7, 7.0)	0.81	10.1	0.00

Figure 1. Number of positive coryza cases by county and production type.



CHARACTERIZATION OF *ENTEROCOCCUS CECORUM* USING WHOLE GENOME SEQUENCING AND EMBRYO LETHALITY ASSAY FOR USE IN AUTOGENOUS VACCINATION

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SUMMARY

Enterococcus cecorum was isolated from multiple tissue sites in 14 diagnostic cases submitted over a 13-month period. An embryo lethality assay was used as an in-vivo assessment of pathogenicity for 87 E. cecorum field strains. There was an overall survivability ranging from 19% to 96% amongst six field strains. Whole genome sequencing followed by single nucleotide polymorphism analysis showed three distinct clusters which coincided with pathogenicity. Analysis of antimicrobial resistance genes present in each of these clusters showed commensal strains to have fewer resistance genes. This analysis showed promising results to use as a tool for selecting strains for autogenous vaccination.

INTRODUCTION

Enterococcus cecorum has been considered part of the normal flora in the intestines of chickens since it was first characterized as a new species in 1983 (1). It is frequently isolated from lesions of the femoral head and free thoracic vertebra characterized by chondronecrosis, and osteomyelitis, primarily in older broilers. Infection of the free thoracic vertebra, by *E. cecorum*, is called *Enterococcal spondylitis* (ES) or Kinky Back (2-4). In younger broilers, infection can cause septic disease manifesting primarily as pericarditis but also can affect the liver and spleen.

Recent increases in the morbidity and mortality of *Enterococcal* infections have led to renewed research in the disease mechanisms of *E. cecorum*. One method to describe pathogenicity in-vivo has been the embryo lethality assay (ELA) (5-8). For this method, bacteria are injected into chicken embryos via allantoic cavity. Mortality is determined by candling and counting non-viable embryos over the next 4 days and survivability curves generated. In one study (3) survivability in broiler embryos for cecal isolates verses isolates of spinal lesions averaged 60% and 23%, respectively.Specific pathogen free (SPF) layer embryo survivability for commensal strains averaged 61% and 9% for pathogenic strains (8).

The development of molecular assays including whole genome sequencing (WGS) have improved the methods by which we characterize bacterial strains. Using WGS, you can begin to look at strain differences, on the molecular level, using the bacterial DNA sequence. This allows scrutiny of specific genes including those for virulence, antimicrobial resistance, or the presence of plasmids and genes associated with their horizontal transfer. There are several schemes for typing and characterizing bacteria utilizing WGS. One such method is the analysis of single nucleotide polymorphisms (SNPs) within core genes, which are those that are present in all genomes (9). A SNP is a change in a single nucleotide base at one point in the sequence occurring in multiple locations throughout the genome but usually do not alter the gene function. Using SNP analysis tells you the epidemiological relatedness of strains, the fewer SNP differences, the more related the strains. A recently published study of E. cecorum comparing commensal cloacal strains to those from clinical lesions using SNP analysis showed two distinct clusters (10).

For this study, we coupled ELA with WGS and SNP analysis to investigate genetic relationships of pathogenicity in strains causing ES, septic disease, and the ability to distinguish them from commensal strains for inclusion in autogenous bacterins.

MATERIALS AND METHODS

Bacterial isolates. Between October 2021 and November 2022, we received 14 diagnostic cases for isolation and characterization of E. cecorum. Clinical signs for most cases were listed as increased mortality, pericarditis, polyserositis, lameness, spondylitis or airsacculitis. One case, submitted for infectious coryza, was included as *E. cecorum* was isolated from the nasal sinus. Table 1 shows the distribution of *E. cecorum* isolation by tissue source. Isolates were obtained using traditional microbiological techniques. Samples were submitted as isolates from external

laboratories, swabs aseptically collected from lesions in the field or as tissues submitted to the Ceva Animal Health laboratory. Samples were struck for isolated colonies on tryptic soy agar containing 5% sheep blood (Hardy Diagnostic) and phenylethyl alcohol agar containing 5% sheep blood (Thermo Fisher). *E. cecorum* identity was confirmed using Rapid ID 32 Strep (Biomeriuex). Isolates were frozen -70±2°C in tryptic soy broth with 25% glycerol for further testing.

Pathogenicity. The pathogenicity of six *E. cecorum* strains was evaluated using ELA methods similar to Borst et al (8). Pure strains were grown overnight in Todd Hewitt Broth (Becton Dickinson) and diluted to contain ~ 102 cfu's per 100 µL. Twenty SPF Layer eggs (Valo) at 12 days of embryonation were inoculated each with ~102 cfu's of *E. cecorum* via allantoic cavity. Eggs were candled twice daily for four days, and mortality recorded. Survivability (Kaplan-Meier) curves were generated using Statgraphics® version 18.1.04.

Molecular analysis. Whole genome sequencing was performed on 87 field strains using Illumina MiniSeq. Raw sequence data for each isolate was normalized and error corrected prior to denovo assembly using SPAdes version 3.15.3 (11). Analysis of SNPs was performed using SNIPPY 4.6.0 (12). Detection of antimicrobial resistance genes of the MEGARES (13) database was done using ABRICATE (14). The core SNP alignment was visualized using Geneious Prime® software version 2022.1.1 to generate a RAxML phylogenetic tree. Reference genomes were downloaded from the National Center for Biotechnology Information (NCBI). *E. cecorum* NCTC12421 NCBI ref.seq. NZ_LS483306 was the reference genome for the SNP analysis. Sequences for commensal strains, NZ_CP010059, NZ_CP010062, NZ_CP010063 and pathogenic strains, NZ_CP010060, NZ_CP010061 and NZ_CP010064 (8, 15) were compared to field strains.

RESULTS

Pathogenicity. The ELA results show distinct differences in pathogenicity amongst the six strains tested. Figure 1 shows the Kaplan-Meier survivability curves. Field strains with the greatest survivability were identified as 4735-5.3 and 4864-2.3, which had a mean survival time of 3.9 days and 3.8 days and survivability rates of 96% and 94%, respectively. The average survival time for the least pathogenic strains was 3.9 days and average survival rate was 95%. Strain 4735-5.3 was isolated from a sinus swab while 4864-2.3 was isolated from liver tissue. Field strains with moderate survivability were identified as 4711-2.2 and 4780-2.4, which had a mean survival time of 3.1 days and 2.9 days and survivability rates of 67% and 63%, respectively. The average survival time for moderately pathogenic strains was 3.0 days and average survival rate was 65%. The site of isolation of 4711-2.2 was unknown and 4780-2.4 was a liver isolate. Field strains with the least survivability were identified as 4735-8.2 and 4780-1.2, which had a mean survival time of 1.9 days and 1.8 days and survivability rates of 31% and 19%, respectively. The average survival time for the most pathogenic strains was 1.9 days and average survival rate was 25%. Strains 4735-8.2 and 4711-2.2 were isolated from sinus and pericardium tissues, respectively. Logrank and Wilcoxson tests showed significant differences amongst the six strains ($P>0.05$). All strains showed some mortality at the first day post inoculation. The most pathogenic strain showed further mortality at 2 days post inoculation.

Molecular analysis. Over all genomes, there were 97,044 core SNPs, producing three distinct clusters of *E. cecorum* in the genomes surveyed. The number of SNPs ranged from 0 – 44,372 with a mean SNP difference of 16,575. The phylogenetic tree of the SNP analysis in Figure 2 color codes the clusters as green suspected to be the least pathogenic, orange suspected to be moderately pathogenic and red possibly the most pathogenic on average, however, no ELA testing has been performed to date on the red cluster strains. Strains that are bolded and italicized are those strains tested in the ELA. Strains that included the two strains least pathogenic to embryos, 4735-5.3 and 4864-2.3, are in the green cluster with a total of 39/87 (45%) of all field strains, as well as the three commensal reference strains. In this cluster, 22/39 (56%) were isolated from liver, heart, or spleen sites. The four strains that are moderately and highly pathogenic in the ELA assay were in the orange cluster containing 29/87 (33%) of field strains. This cluster has 16/29 (55%) of strains isolated from liver, heart, or spleen tissues. The red cluster includes 19/87 (22%) of field strains, including three pathogenic reference strains. This cluster has 15/19 (79%) of strains isolated from vertebral lesions. For strains isolated from systemic sites of liver, heart, and spleen, 22/39 (56%) were in the green cluster and 26/39 (41%) were in the orange cluster. However, for strains isolated from spinal lesions, 15/24 (63%) were in the red cluster, 6/24 (25%) were in the green cluster.

There were 14 total antimicrobial resistance genes found in the genomes tested. Strains possessed from 0 – 10 resistance genes for resistance to tetracyclines, erythromycin, aminoglycosides, lincosamides and streptothrycin. Genes for tetracycline resistance were most common. The presence of antimicrobial resistance genes averaged 3.8, 4.3, and 6.0 genes per strain for green, orange, and red clusters, respectively (Table 2). Decreased susceptibility with more pathogenic strains of *E. cecorum* has been shown in other studies comparing commensal strains to those isolated from diseased cases (16, 17).

DISCUSSION

Differentiating pathogenic from commensal bacteria is important when it comes to vaccine interventions. In this analysis, multiple genotypes of E. cecorum were isolated from similar lesions in the cases reviewed. Additionally, the tissue of isolation does not dictate the degree of virulence or pathogenicity. Regulatory limitations on testing efficacy make selecting the best strain for autogenous vaccination to protect against disease paramount. This study shows that using ELA analysis can identify clear differences in pathogenicity of *E. cecorum* infected embyros .

Genotypes obtained by WGS and SNP analysis appear to show that SNP clustering might be a useful tool to determine pathogenicity or disease association, however, more data needs to be collected before this inference can be verified. Further bioinformatic studies of these genomes could lead to identification of individual virulence factors or groups of genes beneficial for pathogenicity that yield better understanding of Enterococcal disease progression and prevention. The methods used in this study appear to be a useful tool for characterizing *E. cecorum* bydifferentiating pathogenic from commensal strains for inclusion in autogenous vaccination programs.

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Table 1. *E. cecorum* isolates by sample type and tissue source.

	Tissue							
Sample Type	Liver Heart Spleen	Vertebra	Bone Hock Femoral	Sinus	Unknown	Lung	Yolk sac	Grand Total
Swab	42		14	7	3		1	67
Tissue		21						21
Isolate		3	1			1		5
Grand Total	42	24	15	7	3	1	1	93

Figure 1. Embryo lethality assay Kaplan-Meier survival curves.

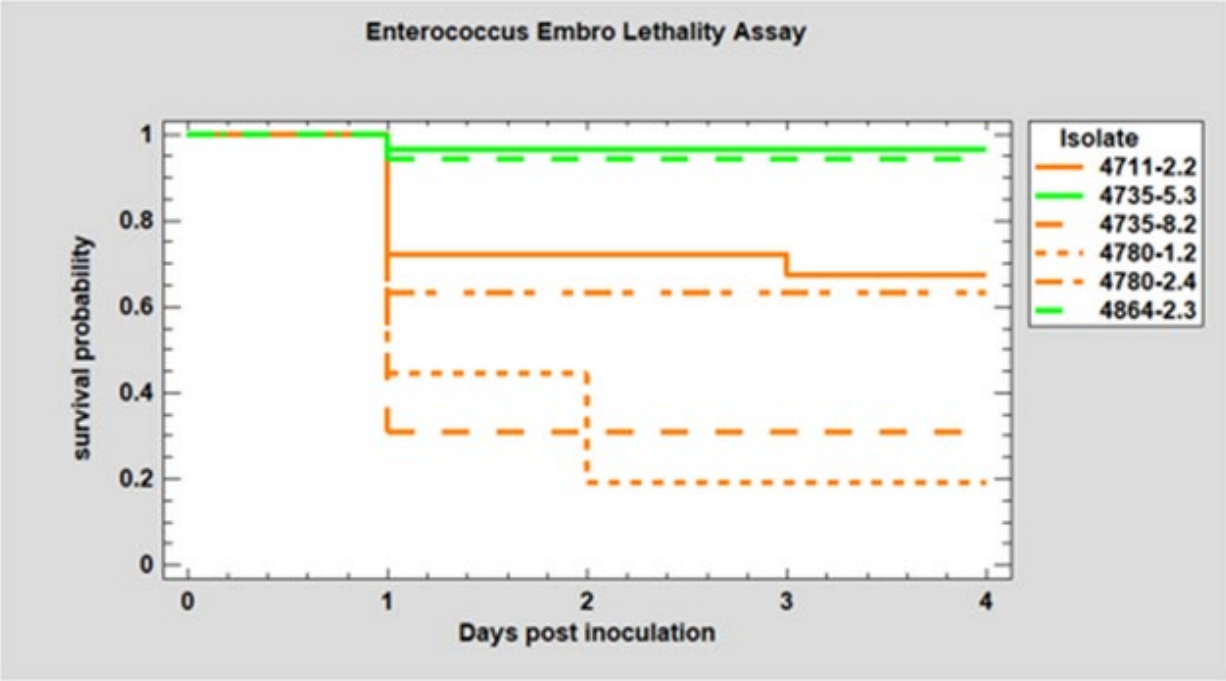

Figure 2. *E. cecorum* RAxML SNP phylogenetic tree.

Table 2. Antimicrobial resistance gene distribution by SNP cluster.

SNP Cluster	TETM	TETL	MEFA	MSRD	ERMG	LNUC	LSAE	LSA	ANT6	ANT9	ERMB	APH2-DPRIME	SAT	LNUG	Average number of genes per strain
green	42	41	15	14	11	3	8	8	4	3	10	1	1		3.8
orange	19	19	5	5	1	1	17	17	19	16	1	3	2	1	4.3
red	22	22	22	22	22	22					1				6.0
Grand Total	83	82	42	41	34	26	25	25	23	19	12	4	3	1	

HIGHLY PATHOGENIC AVIAN INFLUENZA PATHOBIOLOGY IN WILD BIRDS AND POULTRY

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SUMMARY

Highly pathogenic avian influenza subtype H5Nx clade 2.3.4.4b viruses are a major threat to poultry and are spread by wild birds. In December 2021, the virus was detected in the United States (US) in wild waterfowl in North and South Carolina. Since then, the virus has spread to many wild and domestic bird species in the US and many other countries in the Americas. To better understand the disease this virus causes in birds, we conducted experimental studies in chickens, turkeys, and mallards to evaluate the infectivity, pathogenicity, and transmission of one of the early H5N1 HPAI viruses. Differences in clinical signs, mean death time (MDT), and virus transmissibility were found between all three species. The mean bird infective dose (BID_{50}) of the 2022 H5N1 virus was similar in chickens and turkeys ($\leq$3.6 $\log_{10}$ 50% egg infectious doses [EID_{50}]) and the virus transmitted to contact turkeys but not to contact chickens. Infected turkeys took longer to die than chickens, which increased the virus shedding period. The BID_{50} of the H5N1 virus in mallards was <2 $\log_{10}$ EID50 and the virus transmitted to all contacts. Infection in mallards was mostly subclinical, but one duck was lethargic, one died, about 20% developed neurological signs and were euthanized, and 18% developed corneal opacity. These results are important for understanding how this virus spreads.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) viruses, subtype H5 of the A/Goose/Guangdong/1996 (Gs/GD) lineage, have caused multiple intercontinental outbreaks in domestic and wild bird populations through dissemination by migratory waterfowl (3). The H5 HA gene from this lineage has diversified into multiple phylogenetic clades, with the clade 2.3.4.4 viruses becoming predominant. A surge in clade 2.3.4.4b H5Nx HPAI cases occurred in Europe and Asia in the fall of 2020 and outbreaks continue to be reported and are even more extensive than in 2016–2017 and 2020–2021, with H5N1 being the predominant subtype circulating (4). During December 2021 and January 2022, H5N1 HPAI clade 2.3.4.4b viruses were detected in wild birds sampled in the United States (US) in North Carolina and South Carolina (5), becoming the second introduction of Gs/GD clade 2.3.4.4 viruses into the US via wild migratory birds. Since then, the virus has spread through wild birds and has affected more than 57 million domestic birds in 47 States in the US (1-9-23) (6), with unusual persistence of the virus in wild birds and poultry over the summer months (4).

To improve control of H5N1 HPAI in poultry and increase our understanding of the epidemiology and pathobiology of these viruses in domestic and wild bird species, in this study we characterized the infectivity, transmissibility, and pathogenicity of one of the early 2022 North American H5N1 HPAI viruses in chickens, turkeys, and mallards.

MATERIALS AND METHODS

A clade 2.3.4.4b H5N1 HPAIV, A/American Wigeon/South Carolina/22-000345-001/2022 (AW/SC/22), provided by the National Veterinary Services Laboratory, USDA-AHPIS was used in this study. This virus was collected by the Wildlife Services National Wildlife Disease Program, APHIS, USDA. Experiments were performed in a biosafety level-3 enhanced (BSL-3E) facility in accordance with procedures approved by the U.S. National Poultry Research Center (USNPRC) Institutional Biosafety Committee, Agricultural Research Service (ARS), USDA.

All procedures involving animals were reviewed and approved by the USNPRC Institutional Animal Care and Use Committee. A total of 80 chickens (four weeks old), 80 turkeys (three weeks old) and 44 mallards (two weeks old) were used in this study. Experiments were similar to what was used in previous studies (7,8). Birds were divided into groups and inoculated intrachoanally with incremental doses of the virus to determine the mean 50% bird infective dose (BID50) of each virus in each bird species (Table 1). In addition, to evaluate the transmissibility of each virus,

three naïve hatch-mate birds from the same species were added to each dose group 24 hours after inoculation (contact-exposed birds). In all experiments, a group of sham-inoculated birds was also included.

Birds were observed for clinical signs and mortality. Birds showing severe clinical signs were euthanized. Two birds from the groups that received the high doses of the viruses were euthanized and necropsied at 2 days post-inoculation (dpi) and tissues collected for virus quantification, histopathology, and immunohistochemistry using a previously described method (9). Oropharyngeal (OP) and cloacal (CL) swabs were collected from all birds at different time points post-inoculation or contact exposure. Swab and tissue samples were processed for quantitative real-time RT-PCR (qRT-PCR) to determine viral titer equivalents as previously described (10). Sera was collected from all surviving birds to determine the presence of antibodies against AIV using the hemagglutination inhibition (HI) assay (11).

RESULTS

All chickens inoculated with the medium and high dose of AW/SC/22 became infected and died (MDTs between 1 and 2.7 dpi) (Table1). In two chickens in the lower dose group virus was detected in OP swabs but were seronegative at the end of the study. Birds were considered infected if they shed detectable levels of virus at any time and seroconverted at the end of the study. Based on these criteria, the BID50 for this virus in chickens was ≤3.6 log10 50% egg infectious doses (EID_{50}). Virus was detected in OP swabs of contact chickens, but they did not die or seroconvert. No differences in clinical signs were observed in birds inoculated with different virus doses. Chickens that were severely lethargic and/or unresponsive were euthanized. These birds also had ruffled feathers, periorbital swelling, and cyanotic combs. Some chickens died without showing clinical signs (peracute disease). The gross lesions and microscopic lesions observed in the necropsied chickens were typical of HPAIV infection (8).

All chickens inoculated with the high dose of AW/SC/22 shed high titers of the virus by both OP and CL routes at 1dpi (7.7±0.5 and 6.1±0.3 $\log_{10}$ EID_{50} respectively). Chickens that received the medium dose also shed high OP virus titers but took a day longer to peak after which the chickens died. Three of five chickens that received the lower dose of virus also shed high titers. The two remaining birds only shed low titers of the virus by the OP route at 2 dpi. All contact chickens shed low to medium virus titers only by the OP route at 1- and 2-days post contact (dpc).

All turkeys inoculated with AW/SC/22, regardless of the dose given, became infected and died, with MDTs between 2.6 and 4.6 dpi depending on the virus dose (Table 1). The higher the virus dose received, the earlier they died. The BID_{50} was <3.6 $\log_{10}$ EID_{50}. All contact turkeys became infected and died, with MDTs between 3.3 and 5.7 dpc. Most turkeys showed no clinical signs before death. Ruffled feathers and mild lethargy were observed in the rest, which progressed to severe lethargy and prostration at which point they were euthanized.

High virus titers were detected in OP swabs starting at 1 dpi[SE1] from most turkeys inoculated with the medium and high dose of AW/SC/22 (5.9±1.4 and 6.4±0.4 $\log_{10}$ EID_{50}, respectively at 1 dpi). Moderate CL virus shedding was also present in the inoculated birds from the high dose group at this timepoint (4.7±0.6 $\log_{10}$ EID50). Between 2 and 4 dpi, high virus titers were found in CL swabs from all inoculated turkeys in these two groups, which all died by 4 dpi. Shedding in the low dose group occurred later and birds died between 3 and 6 dpi. All contacts shed virus by both routes and as early as 1 dpc in the medium and high dose groups.

The gross lesions and microscopic lesions observed in the necropsied chickens and turkeys were typical of HPAI infection (8,12). Virus antigen was present in respiratory epithelial cells, and parenchymal cells of many organs including cardiac myocytes, hepatocytes, pancreatic acinar cells, microglial cells and neurons, and kidney. Skeletal muscle, lung, spleen, heart, and brain were also collected from the birds necropsied at 2 dpi for viral quantification. Moderate to high virus titers were found in all tissues examined regardless of the bird species.

In mallards, clinical signs started about 2 days later in the lowest dose group and the contact exposed ducks compared to the inoculated mid and high dose groups (Table 1). A total of 14 ducks (41.2%) showed clinical signs. One duck from the mid-dose group was visibly lethargic and had corneal opacity. Neurological signs were observed in seven ducks. One contact transmission duck in the lowest dose group, which showed no clinical signs, died at 7dpc. Six ducks (three inoculated and three contact exposed) developed corneal opacity. At 2 and 4 dpi ducks with neurological signs were selected for necropsy (two ducks each day). Gross lesions included empty intestinal tract, pale livers, friable brain, small thymus, pale pancreas, enlarged heart, petechia in the thigh muscle, and prominent vasculature in the brain. The microscopic lesions and distribution of viral antigen staining were similar between ducks euthanized and examined at 2 and 4dpi, however the lesions were more severe and the antigen staining stronger and more widespread at 4dpi. Microscopic lesions and viral antigen staining were similar to what previously described with H5Nx HPAI viruses (8). The virus titer equivalents, determined by qRT-PCR, were highest in the brain.

Regardless of dose or route of exposure all ducks shed virus in similar patterns. OP shed decreased substantially at 10dpi/9dpc (17%) and 14dpi/13dpc (26%). In contrast, the fewest ducks that were shedding virus by the cloacal

route was 63% at 7dpi/6dpc. At 14dpi/13dpc 65% of the ducks were still shedding virus, although at lower titers. Antibody was detected in serum from all surviving ducks. Therefore, all ducks were infected and the BID50 was <2 log10 EID50.

DISCUSSION

In the present study we found that the 2022 H5N1 HPAI virus was more infectious for chickens and turkeys than previous H5Nx clade 2.3.4.4 HPAI viruses involved in the US outbreak in 2014-2015 (Table 2). A higher mortality was observed in infected turkeys compared to chickens. All turkeys inoculated with AW/SC/22 became infected and died regardless of dose, resulting in a BID_{50} of less than 3.6 $\log_{10}$ EID_{50}. All contact turkeys also became infected and died. The BID_{50} for the chickens was similar as for the turkeys, but not all inoculated chickens died in the lower dose group, and although the virus detected in OP swabs from the contacts, no mortality was observed.

The wild bird index H5N2 and H5N8 HPAI clade 2.3.4.4c viruses detected in the US in 2014 were highly adapted to waterfowl and not yet well adapted to chickens and turkeys based on the comparatively high BID_{50} of these viruses (6, 10, 11). However, H5N2 viruses later isolated in 2015, and after multiple infection cycles in commercial flocks, had higher infectivity for chickens and turkeys (8,12). The virus titers for the more gallinaceous-adapted 2015 H5N2 viruses were similar to what we found with the 2022 H5N1 wild bird isolate, indicating that this virus can more easily infect poultry than the index wild-bird 2014 H5Nx viruses.

One of the big differences in disease presentation between the chickens and the turkeys was the longer MDTs observed in infected turkeys compared to chickens. Unusually long MDTs have also been reported in turkeys infected with other H5Nx clade 2.3.4.4 viruses (12,14). Delay in death while still shedding virus increases the possibility of direct virus transmission or indirect transmission by environmental contamination.

Similar to previous studies (Table 2), the mallards were highly susceptible to infection with the H5N1 virus. All inoculated and contact exposed ducks became infected and were shedding virus at 1dpi. The lower endpoint for the virus was not achieved so the BID_{50} was <2 $\log_{10}$ EID_{50}. The BID_{50} for H5Nx clade 2.3.4.4 viruses have consistently been reported to be <2 log10 EID_{50} in mallards (Table 2). The high quantities of virus shed orally and cloacally, and long duration of cloacal shed, suggest that this virus would spread easily in ducks. Because most don't show clinical disease, they are likely to be productive virus spreaders.

In conclusion, this study highlights the differences in pathobiology between different bird species which affects the epidemiology and ecology of HPAI viruses and has implications in the development of HPAI virus outbreaks.

(The full-length articles of these experiments are being submitted for publication (1,2).)

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Table 1. Infectivity, lethality, and transmission results from chickens, turkeys and mallards inoculated with the H5N1 clade 2.3.4.4 HPAI virus.

Bird species	Dose (log_{10} EID_{50})	Inoculated					Contact exposed			
		# birds shedding virus/total	# dead birds/ total (MDT)[1]	# birds HI positive/ total [2]	# birds infected/ total[3]	BID_{50} (log_{10} EID50) [4]	# birds shedding virus/total	# dead birds/total (MDT)	# birds HI positive/ total	# birds infected/total
Chickens	3.5	5/5	3/5 (2.7)	0/2	0/5	≤3.6	3/3	0/3	0/3	0/3
	5.6	5/5	5/5 (2)	na	5/5		3/3	0/3	0/3	0/3
	7.6	5/5	5/5 (1)	na	5/5		3/3	0/3	0/3	0/3
Turkeys	3.5	4/5	5/5 (4.6)	na	5/5	<3.6	3/3	3/3 (5.7)	na	3/3
	5.6	5/5	5/5 (3.4)	na	5/5		3/3	3/3 (5.3)	na	3/3
	7.6	5/5	5/5 (2.6)	na	5/5		3/3	3/3 (3.3)	na	3/3
Mallards	2	5/5	2/5 (6.5)	4/4	5/5	<2	3/3	1/3 (7)	2/2	3/3
	4	5/5	3/5 (4.3)	4/4	5/5		3/3	1/3 ()	2/2	3/3
	6	12/12	4/12 (4)	12/12	12/12		3/3	0/3	3/3	3/3

na, not applicable

[1]MDT, mean death time, number of dead birds x dpi/total dead birds (expressed as dpi, days post-inoculation or dpc, days post-contact).

[2]Mean HI titers for birds that survived using inoculum virus as antigen. Samples with titers < 3.0 log2 GMT were considered negative.

[3]Inoculated or contact birds were considered infected if they died, shed virus and/or were positive for antibodies at the end of the study.

[4]BID50: 50% bird infective dose

Table 2. Infectivity, lethality, and transmission results from chickens, turkeys, and mallards inoculated with H5Nx clade 2.3.4.4 HPAI viruses.

Bird species	Virus	Dead/total (MDT) 10^6 dose group	BID_{50} (log_{10} EID_{50})	Transmission to contacts
Chickens	A/American Wigeon/South Carolina/22-000345-001/2022 (H5N1)	5/5 (1.4)	≤3.5	No
	A/Northern Pintail/Washington/40964/2014 (H5N2)[a]	3/5 (3.0)	5.7	No
	A/Gyrfalcon/Washington/40188-6/2014 (H5N8)[a]	5/5 (4.1)	4.4	No
	A/turkey/Arkansas/7791/2015 (H5N2)[b]	8/9 (2.1)	5.1	No
	A/turkey/South Dakota/12511/2015 (H5N2)[a]	7/7 (2.2)	3.2	No
	A/turkey/Minnesota/12582/2015 (H5N2)[a]	8/8 (2)	3.6	Yes, in the high dose group
	A/chicken/Iowa/13388/2015 (H5N2)[a]	8/8 (2.4)	3.5	No
Turkeys	A/American Wigeon/South Carolina/22-000345-001/2022 (H5N1)	5/5 (3.2)	<3.5	Yes, with all doses
	A/Northern Pintail/Washington/40964/2014 H5N2[c]	5/5 (5.3)	5.0	Yes, in the high dose group
	A/turkey/Minnesota/12582/2015 (H5N2)[c]	5/5 (5.9)	5.0	Yes, in the high dose group
	A/chicken/Iowa/13388/2015 (H5N2)[c]	5/5 (5.6)	3.0	Yes, in medium and high dose groups
Mallards	A/American Wigeon/South Carolina/22-000345-001/2022 (H5N1)	4/12 (4.0)	<2.0	Yes, with all doses
	A/Northern Pintail/Washington/40964/2014 (H5N2)[b]	0/5	<2.0	Yes, with all doses
	A/Gyrfalcon/Washington/40188-6/2014 (H5N8)[b]	0/5	<2.0	Yes, with all doses
	A/turkey/Minnesota/12582/2015 (H5N2)[b]	1/8	<2.0	Yes, with all doses
	A/chicken/Iowa/13388/2015 (H5N2)[b]	0/8	<2.0	Yes, with all doses

[a]Bertran et al. Virology (11)
[b]De Jesus et al. Virology (6)
[c] Spackman et al. BMC Veterinary Research (10)

PERSISTENCE AND INCIDENCE OF INFECTIOUS BURSAL DISEASE VIRUS IN BROILER FLOCKS IN SASKATCHEWAN OVER A 10-YEAR PERIOD, AND THE EFFECT ON PRODUCTION AND PROCESSING PARAMETERS

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SUMMARY

We have previously reported the immunosuppressive effects of variant infectious bursal disease virus (IBDV) circulating in the Saskatchewan broiler industry in 2009. In this current study, we evaluated 83 broiler flocks in Saskatchewan for IBDV in 2022. We found 63% of the barns evaluated in 2009 were still positive for IBDV in 2022. Additionally, in IBDV positive flocks, there was significantly higher overall condemnations as well as cellulitis, respiratory conditions, ascites and hepatitis condemnations.

INTRODUCTION

Infectious bursal disease (IBD) is a prevalent avian disease worldwide (2,3). Caused by *Birnaviridae* (3), it is further classified into two serotypes; I and II (7). Serotype I are further classified into classical, variant and very virulent and are pathogenic to chickens, usually between 3 to 6 weeks of age (8). Very virulent strains can cause high mortality while variant strains cause lymphoid depletion in the bursa of Fabricius thereby causing immunosuppression (6), the latter being common across Canada (9). This immunosuppression can lead to greater susceptibility to other diseases, reduce weight gain and performance and increase feed conversion and condemnations (4, 5). IBDV is a bi-segmented RNA virus, where segment A encodes viral proteins (VP) 2, 3 and 4. VP2 is the major structural protein (10). The virus is resistant to harsh environments and once in a barn is hard to remove. The main method of control is through vaccination of breeder parents with live or inactivated vaccines to passively transfer maternal antibodies to progeny (3) and/or through vaccination of progeny with live attenuated, recombinant or immune complex vaccines. We previously studied the incidence and economic impact of variant IBDV on broiler flocks in Saskatchewan over a 5-year period, starting in 2009. In order to determine the persistence of IBDV in broiler flocks over a 10-year period, the incidence of IBDV in new barns and the effect on production, we re-evaluated broiler flocks in Saskatchewan.

MATERIALS AND METHODS

Study design. We previously studied the incidence and economic impact of variant IBDV on broiler production in Saskatchewan, Canada between 2007 and 2011 (11), with the majority of barns surveyed in 2009. In 2022, we collected serum, production information (barn age, barn size, total flock mortality and stocking density) and processing information (bird age, average live weight, total number of birds slaughtered, total kilograms slaughtered, total condemnations, condemnation categories and dead on arrivals) from 83 broiler flocks in Saskatchewan.

Serology and incidence of IBDV. Serum was collected from 20 broilers per flock at the time of slaughter, for IBDV and avian reovirus (ARV) enzyme linked immunosorbent assay (ELISA). Both ELISAs were conducted as per IDEXX recommendations at the Animal Health Laboratory, University of Guelph. Flocks with IBDV GM titers of 999 or less were considered negative, while those with GM titers of 1,000 or greater were considered positive. Of flocks considered to be positive, bursa of Fabricius was collected from the subsequent flock from 5 birds per flock between 15 and 20 d of age for polymerase chain reaction (PCR) and VP2 sequencing.

Statistical analysis. Correlations between variables were calculated using Prism 5.0 (GraphPad Software, San Diego, CA). Significance was set at $P<0.05$.

RESULTS

Production information. Production data was collected from 83 broiler flocks (47 premises) in Saskatchewan. Barns were grouped as either new (<10 years of age) or old (>10 years of age). Of the 83 broiler flocks studied, 30

were from new barns, 50 from old barns and 3 were from an undetermined barn age. The average barn size was 15,235 ft2 (range 4,680 to 35,000 ft2). Total flock mortality averaged 6.08% (range 1.5 to 23%) and stocking density averaged 33.58 kg/m2 (range 20 to 40 kg/m2).

Processing information. Processing information was collected at the time of slaughter. Birds were on average 37.5 d of age (range 33 – 46 d of age), with and average live weight of 2.29 kg (range 1.82 to 3.25 kg). On average, the total number of birds slaughtered was 22,086 birds (range 4,986 to 48,389 birds) and 51,279 kg (range 10,421 to 127,747 kg) total weight slaughtered. Total condemnations averaged 1.56% (range 0.08 to 8.19%). Of the total condemnations, the majority were due to cellulitis (0.84%) followed by respiratory conditions (0.29%), ascites (0.18%) and hepatitis (0.10%). Finally, dead on arrivals averaged 0.2% (range 0.03 to 2.04%).

Serology and incidence of IBDV. Serum from 20 broilers per flock was collected for IBDV and ARV ELISA. The average geometric mean (GM) titer was 2,627 (range 1.4 to 12,147) while the average ARV GM was 780 (range 14 to 2,587).

Flocks with IBDV GM titers of 999 or less were considered negative, while those with GM titers of 1,000 or greater were considered positive. The average IBDV GM in negative flocks was 130 (range 1 to 960) and 5,125 (range 1,045 to 12,147) in IBDV positive flocks. The results from VP2 sequencing of bursa of Fabricius showed 56% sampled were 98.3% similar to variant IBDVs SK09 (NC171-like), 11% were 100% similar to variant IBDV SK11 (Del-E-like), 11% were 100% similar to variant IBDV SK12 (586-like), 11% was similar to vaccine IBDV Faragher 52-70 and 11% could not be typed.

Of the barns surveyed in 2009 and again in 2022, 21% which were positive for IBDV in 2009 were no longer in production in 2022, 16% were new barns (<10 year of age) and 63% were positive in both 2009 and 2022.

Statistical analysis. From the data collected, IBDV GM titers were correlated with total condemnations, respiratory, cellulitis, ascites and liver condemnations as well as dead on arrivals, while barn size correlated to ARV GM titers ($P<0.05$).

DISCUSSION

We previously studied the economic impact of variant IBDV infections on broiler production in Saskatchewan (11). In 2009, we found a positive correlation between IBDV antibody titers and bursal atrophy. Similarly, the results from our current study showed variant IBDV strains circulating at Saskatchewan broiler farms are similar to SK09 (NC171-like), SK11 (Del-E-like) and SK12 (586-like) which is consistent with the data from 2007 and 2009 (1,11). In this study we found 28% of barns were positive for variant IBDV compared to 43% in 2009. These barns were older than the barns with negative IBDV flocks, and had higher than average total flock mortality, total condemnations, including condemnation categories of cellulitis, respiratory conditions, ascites and hepatitis. This is consistent with our 2007 study, which showed hepatitis condemnations were correlated with IBDV titers indicating hepatic condemnations were associated with immunosuppression due to variant IBDV and not a primary bacterial pathogen (1). The persistence and similarity of variant IBDV infections in Saskatchewan between 2007 and 2022, indicate the resistance of the virus to cleaning and disinfecting, once entering a barn it is difficult to remove. Of the barns surveyed in 2009 and 2022, 21% were no longer in production in 2022, 16% were new barns (<10 years of age) positive for IBDV, and 63% were positive in both survey periods. The circulating variant IBDVs in Saskatchewan are not consistent with IBDVs in currently available vaccines, and the persistence indicates these vaccines are likely not effective in controlling infections. More work is needed regarding the relationship and significance of ARV titers to production and processing parameters collected in this study.

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A NOVEL BROILER CHICKENS LEG PROBLEM: SEVERE MYOSITIS CAUSED BY *ENTEROCOCCUS CECORUM* AFFECTING MEDIAL THIGH MUSCLES

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SUMMARY

Over the last two years, the isolation of *Enterococcus (E.) cecorum* from broiler cases at the Poultry Research and Diagnostic Laboratory (PRDL) of Mississippi State University has increased exponentially. These cases have been associated with high mortality, lameness, and septicemia. *E. cecorum* has been isolated from the free thoracic vertebra, pericardium, femoral head, liver, and hock joint. In addition, an increase in the presentation of gross lesions in the thigh medial muscles has been observed recently. These findings are causing significant welfare, productivity, and financial concerns. Microscopic examination of the affected muscles describes mild to severe hemorrhagic myositis, with myodegeneration and the presence of intralesional bacteria. Pure cultures of *E. cecorum* exhibiting heavy growth have been isolated from affected muscles. Due to the persistence of this finding, samples of the leg's muscles are being collected bacteriological and microscopic examinations are collected when macroscopic lesions are present.

INTRODUCTION

Leg broiler chicken problems have always been a significant concern for the poultry industry. Although these problems do not cause high mortality, they significantly affect the chickens performance because the birds cannot move and consequently cannot reach the feeders and drinkers. For this reason, there is a drop in feed consumption that will later be reflected in low weight gain, inadequate feed conversion, and an increase in the number of culled chickens. Leg problems can also be related to food safety issues because of the lack of uniformity. Processing steps such as de-feathering and evisceration get affected when the size of the carcasses is not similar. Additionally, down chickens will be in constant contact with the litter, so there will be a significant increase in the presence of dirty feathers, conditions that favor the proliferation of bacteria important in terms of public health, such as *Salmonella* spp. and *Campylobacter* spp.

Leg problems origin could be multifactorial. Some etiologies affect the tendons, others can affect the bones and/or muscles. Infectious diseases such as viral arthritis (avian reovirus), and septic arthritis by *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*, among others, are extremely common in broiler chickens. On the other hand, nutritional deficiencies can impair the birds' movement because of problems in the bones and muscles (biotin, calcium, and phosphorus deficiencies), and because of the presentation of nervous signs (encephalomalacia by vitamin E deficiency).

This case report explores the characteristics of two broiler chicken cases with leg problems received at the PRDL in 2022, from a broiler chicken company in the State of Mississippi.

Case 1. Eight live and two dead, 24-day-old broilers. Live birds leg problems such as reluctance to move, extended legs, and birds resting on their hocks with their feet and shanks raised. Reported three days mortality was as follows: 31, 51, and 55 birds per day. The main company claim was that the birds were getting down and dying.

Case 2. Ten alive, 14-days old broilers. Main clinical signs observed in all birds included reluctance to move, depression, and huddling. Reported last three day mortality was as follows: 151, 214, and 217 birds per day.
The flock average size in these two cases was 21.000 to 23.000 broilers per flock.

MATERIALS AND METHODS

For both cases, necropsy was performed following PRDL procedures, identifying the main gross lesions and collecting samples according to the changes observed in the affected organs. Samples for bacterial culture were streaked on Blood Agar, CNA-Columbia, and MacConkey Agars. A pool of organs was inoculated in tetrathionate broth (*Salmonella* enrichment) (Remel™ Thermo Fisher Scientific™). Bacteria identification was performed after 24

hours of incubation at 37°C, under aerobic conditions by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Vitek® MS instrument (bioMerieux, Inc). *Salmonella* spp was genotyped by Intergenic Sequence Ribotyping (ISR). Due to the observed leg problems samples of tendons were collected for Avian reovirus isolation (cell culture in LMH cells). Due to the high mortality reported in case 2, oropharyngeal swabs were collected to perform Avian Influenza RT-PCR. Pieces of leg muscles (thigh) were collected for histopathology.

RESULTS

Necropsy Case 1. Most of the broilers presented fibrotic and hard gastrocnemius tendons. Scratches on the flank and footpad dermatitis score 1 were present in half of the chickens. Septicemic lesions such as pericarditis and airsacculitis were observed in most of the examined birds. Hock joints were affected presenting exudates from serous to fibrinopurulent. Knee joints had similar exudates. Septic arthritis and femoral head necrosis were observed in 25% of examined chickens. Cellulitis was observed in one bird, and subcutaneous edema was present in the broilers dead on arrival. Interestingly, half of the birds presented yolk sac retention. One chicken exhibited caseous exudate and deformation and displacement of the fourth thoracic vertebra.

Necropsy Case 2. Tenosynovitis was the gross finding with the highest prevalence in these birds. Fibrosis was observed in the gastrocnemius muscle and digital flexor tendons. Septicemic lesions such as multifocal hepatitis with lesions concentrated on the caudal margins of the liver, pericarditis, and mild airsacculitis were observed in three chickens. Like case 1, some chickens presented yolk sac retention. Other gross findings like proventriculitis, sternal hematoma, sternal bursitis, femoral head necrosis/osteomyelitis, and footpad dermatitis score 1 were also observed at least in one chicken.

Muscular lesions. In both cases, the muscles of the medial thigh area (flexor cruris medialis, adductor profundus, and femoro-cruralis) were affected, exhibiting marked myositis with the presence of hemorrhages, edema, and fibrin. Muscle rupture was also observed.

Bacterial and virus isolation Case 1. *Enterococcus cecorum* (heavy growth) was isolated from hock joints, femoral head necrosis, muscle, and pericardium. A slight growth was observed in the sample collected from the vertebra. *Salmonella* spp was isolated after enrichment. This isolate was identified as *Salmonella* Infantis genotype CP019202, by ISR. Avian reovirus was detected in the first passage of LMH cells.

Bacterial and virus isolation Case 2. *Enterococcus cecorum* (heavy growth) was isolated from liver, pericardium, femoral head, and thigh muscles. All collected samples were negative for *Salmonella* spp. Avian reovirus was not detected in samples of tendons in this case.

Avian influenza RT-PCR Case 2. Negative.

Histopathology. Skeletal muscle had mild interstitial edema and acute multifocal hemorrhages. Mild to moderate histiocytes and heterophils infiltration was observed in the interstitium. A few myocytes have pale to hypereosinophilic sarcoplasm with loss of cross striations. In the more severely affected section, basophilic stippling (interpreted as early mineralization) was present.

Final microscopic diagnostic: mild to moderate heterophilic and histiocytic myositis, with myodegeneration, myonecrosis, minimal mineralization, interstitial edema, and hemorrhage.

DISCUSSION

The emergence of *E. cecorum* causing systemic disease must be considered important in terms of poultry health, and because of the severe impact on the productive performance of the affected chickens. It is known that this bacterium can cause high mortality and high morbidity, and its tropism for cartilage results in marked leg problems because of the presentation of femoral head necrosis and synovitis. However, the muscle damage observed in these two cases was not documented before, this condition along with the commonly observed femoral head necrosis and hock joint arthritis is contributing to the frequent report of leg problems. So, these findings suggest E. cecorum must be considered involved in leg problems affecting not only the bones but also the muscles. Therefore, this bacterium causing severe myositis on medial thigh muscles not only affects the broiler's performance and welfare but is a potential cause of leg quarters condemnations.

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ELEVATED MORTALITY WITH BLOODY INTESTINES IN A COMMERCIAL TURKEY FLOCK

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SUMMARY

Four 10-week-old commercial turkeys with a history of elevated mortality was submitted to the California Animal Health and Food Safety Laboratory System (CAHFS), Turlock branch for postmortem evaluation. On gross examination, there was severe hemorrhage throughout the intestinal tract and spleens were markedly enlarged and mottled. Microscopically, numerous viral intranuclear inclusions were observed in the spleen and intestine, and there was intestinal mucosal necrosis with colonies of rod-shaped bacteria. Large numbers of *Clostridium perfringens* were isolated from the intestine, and hemorrhagic enteritis virus (HEV) infection was confirmed via rtPCR on the spleen. This report highlights a severe case of HEV followed by secondary necrotic enteritis infection in a commercial turkey flock in California.

INTRODUCTION

Necrotic enteritis (NE) is a serious health problem for the commercial chicken and turkey industry globally resulting in economic losses due to elevated mortality, poor feed conversion, decrease weight gain and increase medication cost (1). The etiologic agent of NE is *Clostridium perfringens*, a gram-positive anaerobic bacterium that is ubiquitous in the poultry house environment and a common commensal in the bird's gastrointestinal tract (2).

Immunosuppressive agents such as viruses and mycotoxins can predispose poultry to NE outbreaks as well as other secondary bacterial infections. In turkeys, hemorrhagic enteritis virus (HEV), a turkey adenovirus 3 (TAdV-3) of the genus *Siadenovirus* is an important immunosuppressive virus (3). We describe herein the clinical history, gross lesions and microscopic lesions of a severe case of necrotic enteritis and concurrent hemorrhagic enteritis virus infection in a commercial turkey flock in California.

MATERIAL AND METHODS

Four 10-week-old commercial turkeys with a history of elevated mortality were submitted to the CAHFS, Turlock branch for postmortem evaluation. The submission included 3 live and 1 dead. Live birds were euthanized humanely using carbon dioxide. A complete postmortem evaluation was performed with samples taken for serology, microbiology, histopathology and reverse transcription quantitative PCR (RT-qPCR).

At necropsy, tissue samples were collected from the four birds, immersion fixed in 10% neutral buffered formalin for 24-48 hr, paraffin-embedded and processed routinely producing 4µm thick hematoxylin and eosin-stained slides. Intestinal mucosa was swabbed and inoculated onto Brucella agar with 5% sheep blood and incubated at 37°C anaerobically. Testing for avian influenza virus (AIV) by RT-qPCR on oropharyngeal swab pools was performed (CAHFS, Davis; National Animal Health Laboratory Network protocol) (4). Molecular diagnostic detection of HEV was performed by PCR on pooled splenic tissue samples (5). ProFLOK HEV-T Ab (Zoetis, Kalamazoo, MI, USA) rapid serologic ELISA testing for the detection of pre and post vaccination HEV antibodies was performed on 6µL of serum from each live bird.

RESULTS

On gross examination, there was severe hemorrhage throughout the intestinal tract and spleens were markedly enlarged and mottled. Microscopically, numerous large viral intranuclear inclusions were observed in the spleen and intestine, and there was intestinal mucosal necrosis with large numbers of rod-shaped bacteria. Large numbers of *Clostridium perfringens* were isolated from the intestine, and HEV infection was confirmed via rtPCR on the spleen.

DISCUSSION AND CONCLUSION

Concurrent infections of HEV and NE is turkeys may be linked to numerous factors related to the host, the environment and the pathogens. Moreover, production, management practices and stressors influence the severity of disease and resulting economic losses. HEV is considered endemic in commercial turkey flock in the Central Valley of California and the immunosuppressive effect of the virus had been associated with increase infections by bacterial pathogens such as *E. coli* and *Clostridium* species (6, 7). Disease control is based on strict biosecurity, farm cleaning and sanitation, good husbandry practices and appropriate vaccination.

(The full length article will be published in *The Journal of Veterinary Diagnostic Investigation*.)

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ASSESSING THE INNATE IMMUNE RESPONSE IN CHICKENS

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SUMMARY

Innate immunity is characteristically nonspecific, rapid and the very first line of immune defense. It is also paramount in initiating the adaptive immune response. There are several immune cells that contribute to innate immunity which include leukocytes such as neutrophils (heterophils in avian species), monocytes, macrophages and natural killer cells. These cells are involved in phagocytosis, antigen presentation, free radical and cytokine production. Typically, the methods for measuring the innate immune response are very technical, expensive, and labor intensive. We have developed an assay whereby peripheral blood cells are stimulated with LPS to release the cytokine, IL-6. IL-6 is then measured using an ELISA. This method has proven to be a repeatable, direct method to measure the innate immune response in a time and cost-efficient manner and should prove to be a useful tool in assessing the innate immune response in poultry.

INTRODUCTION

A bird's immunity can be enhanced by using vaccines and adjuvants to stimulate the adaptive component of the immune response leading to increased humoral (i.e., antibody) and/or cell mediated responses. Using serologic assays to measure antibody levels is a useful, convenient and cost-effective method to evaluate the adaptive (i.e., humoral) immune response. An equally important component of the bird's immune system is the often-unnoticed innate immune response. Innate immunity is characteristically nonspecific, rapid and the very first line of immune defense. It is also paramount in initiating the adaptive response.(1) There are several immune cells that contribute to innate immunity which include leukocytes such as neutrophils (heterophils in avian species), monocytes, macrophages and natural killer cells. These cells are involved in phagocytosis, antigen presentation, free radical and cytokine production.(1) Typically, the methods for measuring the innate immune response are very technical, may require elaborate and expensive equipment, and labor intensive. Such methods include separating cells and using cell surface markers and subjecting the cells to a fluorescent-activated cell sorting (FACS) technique using a flow cytometer, using upregulation of mRNA and/or evaluating the amount of super oxide anion released as a result of inducing an oxidative burst. We have attempted some of these methods with limited success. The objective of this study was to develop a reliable, cost and time efficient method to assess the innate immune response in chickens that could be easily performed without specialized equipment.

Recently, we have had success in determining the innate response by stimulating blood cells with lipopolysaccharide (LPS) derived from a strain of *Salmonella* spp. bacteria and measuring the amount of interleukin-6 (IL-6) cytokine released using an enzyme linked immunosorbent assay (ELISA). When optimized, we believe this technique will be an invaluable tool to allow us to explore ways in which to enhance the innate immune response.

MATERIALS AND METHODS

Birds and blood samples. Adult male specific pathogen free (SPF) layer type chickens were hatched (SPF eggs obtained from VALO BioMedia, Adel, IA, USA) and reared in our biologic containment 2 facility. Birds used in this study were hatch mates. Blood was collected from the wing vein into a syringe containing sodium heparin. Following collection, the heparinized blood was kept at room temperature and used within 30 mins of collection.

Media and reagents. Ross Park Memorial Institute (RPMI) 1640 media was obtained from Corning, Inc. (Manassas, VA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for assaying IL-6 were obtained from MyBioSource, Inc. (San Diego, CA) and used as per manufacturer's instructions. Salmonella Minnesota LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA). Histopaque-1119, density: 1.119 g/mL (Sigma-Aldrich, St. Louis, MO, USA) was used for cell separation procedures.

Cell separation procedures. Heparinized chicken blood was layered onto an equal volume of Histopaque-119 separation media. This preparation was centrifuged at 400 x g for 30 mins. Top layer, middle band, and bottom layer were separated and pelleted at 1700 x g for 5 minutes, then resuspended in RPMI 1640.

Innate immunity assay procedure. Blood was collected from birds into syringes containing sodium heparin as indicated above and the cells separated on Histopaque-119 separation media. The cells were counted and then activated with LPS. Following activation, the cells were centrifuged, the supernatant removed and assayed for IL-6 using the IL-6 ELISA kits. The results were statistically analyzed using the student's t test.

RESULTS AND DISCUSSION

Following numerous trials to optimize such parameters as cell numbers, LPS activation times, separation procedures, etc., it was found that the stimulation of cells form the separation bands containing primarily peripheral blood mononuclear cells (PBMCs) had statistically significant higher IL-6 values than those of corresponding PBS stimulated (i.e., negative controls) cells. The trials were repeated several times with each time yielding statistically significant results indicating high confidence for repeatability. This assay represents a relatively easy, straightforward, and direct method to measure the innate immune response in a time and cost-efficient manner and should prove to be a useful tool in assessing the innate immune response in poultry.

Trained innate immunity is a term used to describe the concept of enhancing innate immunity through immunological memory once thought to occur only with the adaptive immune response.(2, 3). Interest in enhancing innate immunity has increased in recent years as a measure to control infectious diseases such as *Salmonella* Enteriditis(4). The trained innate immune response has been demonstrated in chicken PBMCs using various substances including LPS, beta-glucan, IL-4 and combinations of the aforementioned.(5, 6) The ability to train the innate immune response may be an important factor in future vaccine and vaccine adjuvant development and in developing vaccination programs.

(Note: The full-length article will be published in the International Journal of Veterinary Science and Medicine)

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PREVALENCE AND VIRAL ECOLOGY OF AVIAN INFLUENZA IN WETLANDS OF HIGH VERSUS LOW WATERFOWL ABUNDANCE IN THE CENTRAL VALLEY OF CALIFORNIA

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ABSTRACT

Exposure to highly pathogenic avian influenza (HPAI) is a significant concern to the commercial poultry industry. Over the course of 2022, outbreaks of HPAI have resulted in the loss of millions of domestic birds. Waterfowl are the primary reservoir of the virus, therefore understanding the presence/absence and type of Avian Influenza (AIV) in waterfowl and their habitat is essential toward both understanding and mitigating risk. Here we compare the presence/absence and viral ecology of AIV in abiotic reservoirs of both high and low waterfowl density wetlands in the summer of 2021. Specifically, sets of oropharyngeal and cloacal swabs of live waterfowl, sediment, and wetland water samples were collected and processed via whole-segment amplification and long-read sequencing from both high and low abundance wetlands as detected by NEXt generation RADar (NEXRAD) and human observation. Results showed no statistical difference between AIV detection in high versus low waterfowl abundance wetlands with respect to biotic reservoirs of water (40.82% (n=49) vs 28.89% (n=45), p = 0.3202) and sediment (89.80% (n = 49) vs 77.78% (n=45), p = 0.191). However, the overall trends suggest greater prevalence of virus in higher abundance wetlands as suggested by the above described data. Prevalence in collected bird swabs alloted to 48.96% positive samples within selected high use areas. This data gives insight on the high sensitivity of testing for AIV within potential environmental abiotic reservoirs.

INTRODUCTION

Migratory waterfowl have been found to represent an asymptomatic, naturally occurring reservoir for AIV, which can be spread to commercial poultry (1). After the first case of a Eurasian strain of HPAI was observed in the United States in January 2022, wild bird surveillance has increased dramatically (2). In addition, as the primary reservoir, waterfowl are considered the primary source of novel strains of AIV due to the crossover of migratory routes and the common exchange of the virus from bird to bird (3).

Commercial poultry are highly susceptible to both high-pathogenic and low-pathogenic strains of AIV (4). To this point in the outbreak, 58.24 million poultry birds from 748 domestic flocks have been killed by or euthanized due to HPAI infections (5). Current surveillance focused on historic distribution maps is limited in that it does not account for ephemeral changes in migratory paths due to changes in roosting habitat and feeding locations (6).

Remote surveillance of waterfowl including NEXRAD radar offers a novel method of understanding waterfowl distribution and hence potential risk of disease transmission to domestic poultry. Applications such as the Waterfowl Alert Network (WAN), which utilize remote technologies including NEXRAD radar offer a potentially useful tool as a real-time tool for commercial producers (7). As an example, 11 GPS-tagged ducks between 2015-2020, were observed to have spent substantial amounts of time near (i.e. within 10 km) or on commercial livestock or poultry facilities (8).

In the central valley of California, peak migrations of waterfowl occur between late fall and early spring as part of the normal migratory path of the Pacific Flyway (9). Next generation surveillance tools such as the WAN could be used to identify high abundance waterfowl roosting habitat in close proximity to commercial poultry. Here we describe the sampling of both biotic and abiotic viral reservoirs in high and low waterfowl density wetlands, in order to better understand the relationship between the presence/absence of AIV in low waterfowl abundance versus high waterfowl abundance wetlands. Specifically, a combination of NEXRAD surveillance using the WAN and human observation of waterfowl were used to guide collection of biotic and abiotic samples (e.g. sediment and wetland water) as described by McCuen et. al (10). Understanding the differences in prevalence and viral ecology between high and low use

wetlands and their associated waterfowl is fundamental toward understanding the viral distribution and ecology of HPAI in wetland environments in close proximity to commercial poultry.

MATERIALS AND METHODS

Wetland selection criteria. Water samples for the pre-migration time frame were collected in August and September 2021, from 10 individual wetland ponds from each of 2 watershed basins (5 high-use and 5 low-use), totaling 20 wetland ponds identified for sampling (Figure 1). Within each of the 2 basins (specifically Sacramento National Wildlife Refuge Complex and the California Butte Sink State Wildlife Areas), locations were chosen due to waterfowl use within each individual pond. In 2021, ponds were identified for sampling using telemetry data (home ranges of ducks in summer and winter) and satellite imagery that indicated habitats that were flooded from late July to early September 2021. Additional insights were gained from the federal wildlife refuge and state wildlife area staff.

The selected wetlands were free of chemical treatments of any kind, in order to represent a natural habitat for waterfowl. Potential salinity was also a factor in determining sampling location, as AIV prevalence is typically lower in brackish environments (11). In the selected high-use ponds, sampling locations were chosen due to visual waterfowl use (molted feathers, birds visually seen, feces left behind, etc.) as well as knowledge of historical trapping sites. In low-use ponds, sampling sites were chosen on similar criteria; although if there was no physical sign of waterfowl, sampling locations were replicated in a similar fashion to those within the high-use ponds (e.g., similar water depth, distance from pond/island edge, and aquatic vegetation).

Sample collection. Samples were collected below the water's surface, midway down the water column, with no surface water allowed in the 10-liter carboy. Measurements of pH, temperature, dissolved oxygen, and conductivity were recorded with the YSI Professional Plus sensor at each of the five locations within an individual wetland pond, at the same depth water samples were taken from. Along with YSI meter readings, other physical environmental variables were also recorded including water depth, soil sample depth and distance from the water's edge, emergent and submergent vegetation, as well as whether or not the area was shaded. We also recorded/estimated the numbers of each bird species observed upon arrival at each pond for sampling. At each of the five locations, a 10-liter water sample was collected according to the lower limit of large volumes considered to be adequate for determining pathogen presence in water (12). A 15 mL surface water sample was collected in a 15mL conical tube from each wetland pond to compare with previous sampling methods. One 15 mL sediment sample was collected in a 15 mL conical tube at each individual sampling site within a wetland to compare the presence and persistence of AIV in sediment to water samples. Sediment samples were collected 1 meter or less from the water's edge, with a nearby correlation to the water sample locations. Sediment samples were obtained at the level of the water table. GPS coordinates for all sampling sites have been recorded, in order to retain accuracy for the sampling of the overwintering and migration period involving future sampling periods. 10-liter water samples were kept on ice and taken back to the lab, where they were stored in a 4°C walk-in fridge, for next-day filtration. Surface water and soil samples were frozen at -80°C to preserve viral integrity prior to RNA extraction. To compare the potential AIV findings from the water and sediment samples, an aimed quota of at least 10 sets of cloacal and oropharyngeal swab samples from live waterfowl was desired to be met from ponds where trapping efforts are being conducted from federal agency partners (USFWS and USGS).

Filtration methods. Conventional tangential flow ultrafiltration separates solutes that differ by tenfold in size through membrane pore size, qualifying this method of filtration as an appropriate approach for AIV detection in larger volumes of water (13). Viral particles were retained by molecular weight cut-offs and concentrated in the retentate while molecules smaller than the filter's pore size flowed through the membrane (14). Prior to filtration, each filter was primed with 500 mL of 0.1NaPP/1L Deionized water. Each 10-liter carboy was filtered using individual Asahi-Kasei Rexeed 25s columns. Each 10-liter carboy was filtered down to a 15 mL retentate to be comparable with the 15 mL unfiltered surface water sample. Once viral particles were caught in the filter membrane, the excess permeate flowed out of the system and into a permeate reservoir, where it was later discarded. Pressure of the filtration system did not exceed 20 psi, and typically ran between 10-15 psi. Upon completion, each filter was eluted with a 500 mL solution of (0.1g NaPP, 10mL 0.01% Tween 80, 10mL 0.001% Antifoam)/1L Deionized water, in order to flush the virus from the membrane into the retentate solutions to be captured. Final samples were immediately placed in a -80°C freezer to preserve viral integrity, until RNA extraction was performed.

PCR and sequencing. RNA from water and swab samples (from wild caught birds) were extracted using the AllPrep PowerViral DNA/RNA (QIAgen). Following extraction (immediately after extraction for water samples), we subjected RNA samples to Influenza A virus whole-segment amplification using multi-segment RT-PCR (15). This procedure uses primers that are complementary to genome segment packaging regions (uni12 and uni13), which are conserved among all influenza A viruses, including AIV. Thus, this procedure amplifies entire gene segments if they are present in the sample. We conducted gel electrophoresis on select samples to confirm genome segment

amplification. Amplicons were barcoded in a second PCR using the Oxford Nanopore PCR Barcoding Kit (Oxford Nanopore Technologies), then multiplexed and prepared for sequencing on R10.4 flow cells (aka Q20+) on the MinION sequencer (Oxford Nanopore Technologies). The MinION sequenced single DNA molecules and allowed for the recovery of entire influenza genome segments (16; 17). We reference this method as amplification/sequencing hereafter.

Bioinformatics analyses. Output from the MinION sequencer was analyzed using a custom pipeline that is openly available online (10, 18). Briefly, raw signal files (.fast5 format) were base-called using Guppy in high accuracy base calling mode (HAC). After quality filtering using Nanofilt (19), reads were demultiplexed (i.e. assigned to a sample) and primers trimmed using Guppy, with both barcodes matching. We used a single brand-new flow cell and included negative and positive controls throughout the sample workflow. NCBI command line BLAST using GNU Parallel (20) was used to search demultiplexed files against all avian influenza whole genome sequences available in the NIAID Influenza Research Database (IRD) (21). Sample metadata from IRD was used to annotate likely subtypes and hosts of AIV sequences detected in each collecting location, based on the closest match in the IRD.

RESULTS

Sixty water samples and 50 soil samples from identified high and low use wetlands for the pre-migration sampling period were collected in the months of August to mid-September of 2021 (Figure 1). From live birds within select high use wetlands, 182 sets of oropharyngeal and cloacal swabs were collected as well, in order to compare the AIV ecology and prevalence to that in the environment (Table 1). For designated high-use wetland areas, the amount of waterfowl observed was, on average, multiple hundreds; in low use ponds the number of waterfowl was typically less than 25.

Avian influenza reads within water samples. Of the 120 environmental water samples (100 filtered and 20 non-filtered), 94 have undergone whole-segment amplification and long-read sequencing. Within these analyzed samples, 30 filtered and 3 non-filtered samples had sequence reads matching AIV within the NIAID IRD, leading to an overall positivity rate of 35.11%. Comparisons thus far between high and low use wetlands show a trend regarding higher viral abundance in high-use areas over low-use areas (40.82% prevalence vs 28.89%), but the differences within this data set so far are statistically insignificant after preliminary proportional tests ($p = 0.3202$) (Figure 2a).

Avian influenza reads within sediment samples. Of the 100 collected soil samples, 94 have been analyzed. Of these 94 analyzed samples, 80 have yielded reads matching AIV within the NIAID IRD, giving a positive testing rate of 85.11%. Of these 80 positive samples, 6 have contained full genomes, and 13 others containing 7 of 8 AIV segments. Similar to the aforementioned water samples, the difference between high and low use wetlands is statistically insignificant using the same proportional testing ($p = 0.191$). However, a trend still appears to be favoring high-use areas over low use areas (89.80% vs 77.78%, Figure 2b).

Avian influenza reads within live bird swabs. Of the 96 bird swabs analyzed from high waterfowl abundance wetlands, 48.96% (47/96) had sequences consistent with AIV per the NIAID IRD. Within these positive samples, 4 complete AIV strains were identified through complete genomes being collected as well as partial genomes of 4 other AIV strains, showing a high amount of viral ecology within the biotic reservoir of the virus.

DISCUSSION

Waterfowl migrations have been tied to AIV outbreaks in poultry, especially as migrations peak and birds congregate (22). Therefore, understanding the relationship between waterfowl and their habitat with respect to AIV prevalence and viral ecology could provide new insights to commercial poultry farms with respect to AIV risk analysis. AIV persistence in the environment has been documented, but continues to be not fully understood with respect to HPAI disease transmission. Previous studies have shown AIV retains infectivity in sediment up to 2 days after the host expires, but viral nucleic acids remain up to 21 days afterwards (23). AIV also has been found to withhold higher persistence in sediment over duck feces (24). Of the 94 respective soil and water samples analyzed in this study, sediment has a much higher prevalence rate than that of aqueous samples (85.11% prevalence vs. 35.11%). However, this study has shown a higher rate of positivity in aqueous samples than previous studies, in line with McCuen, et al (10).Within previous studies, AIV has been isolated in natural aqueous environments under certain environmental parameters such as low temperature, higher than neutral pH values, and lower salinity (25). In this study we have isolated viral segments in water samples from both high and low use wetlands within environmental parameter ssuch as temperature and salinity values, upwards of 29.4℃ and 1617 μS/cm respectively (Table 2). Once sub- and

genotyping analysis has been completed, comparisons of viral ecology between biotic and abiotic samples can be further analyzed.

Swab sets from live birds were exclusively collected within high use wetlands; due to ongoing partnered trapping efforts, no birds were captured on low use study areas. The development of targeted surveillance of wild waterfowl populations and their environment remains an active goal for the global poultry industry (26).

Next generation remote sensing tools such as the Waterfowl Alert Network can aid in the monitoring of waterfowl abundance in close proximity to commercial poultry. Here the Waterfowl Alert Network is used to identify wetlands with high and low waterfowl abundance in order to compare the viral ecology of the environment (i.e. sediment and wetland water) to the AIV found in waterfowl associated with both high- and low-density wetlands. If environmental testing continues to prove successful, a continual surveillance program linking the Waterfowl Alert Network (WAN) to targeted environmental testing could be established, in order to better understand risk to commercial poultry facilities. In one scenario, the combination of the WAN and testing could be used to inform farmers that waterfowl are present or nearby. That information could be used to drive waterfowl away from commercial facilities, as well as heighten biosecurity when a larger threat is present. Other pathways attempting to keep waterfowl off of, and a distance from, poultry facilities have been found to be somewhat successful (such as the utilization of class-III B qualified lasers to scare off aerial birds), but never completely effective (27). It is clear that on a global scale, there is a significant need for next generation applications like the WAN (22; 26; 28; 29). With systems like this, in cooperation with environmental sampling, as well as sampling from wild birds themselves, the persistence and ecology of AIV in waterfowl populations can be understood, and help reduce the chances of potential outbreaks.

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Figure 1. Sampling locations.

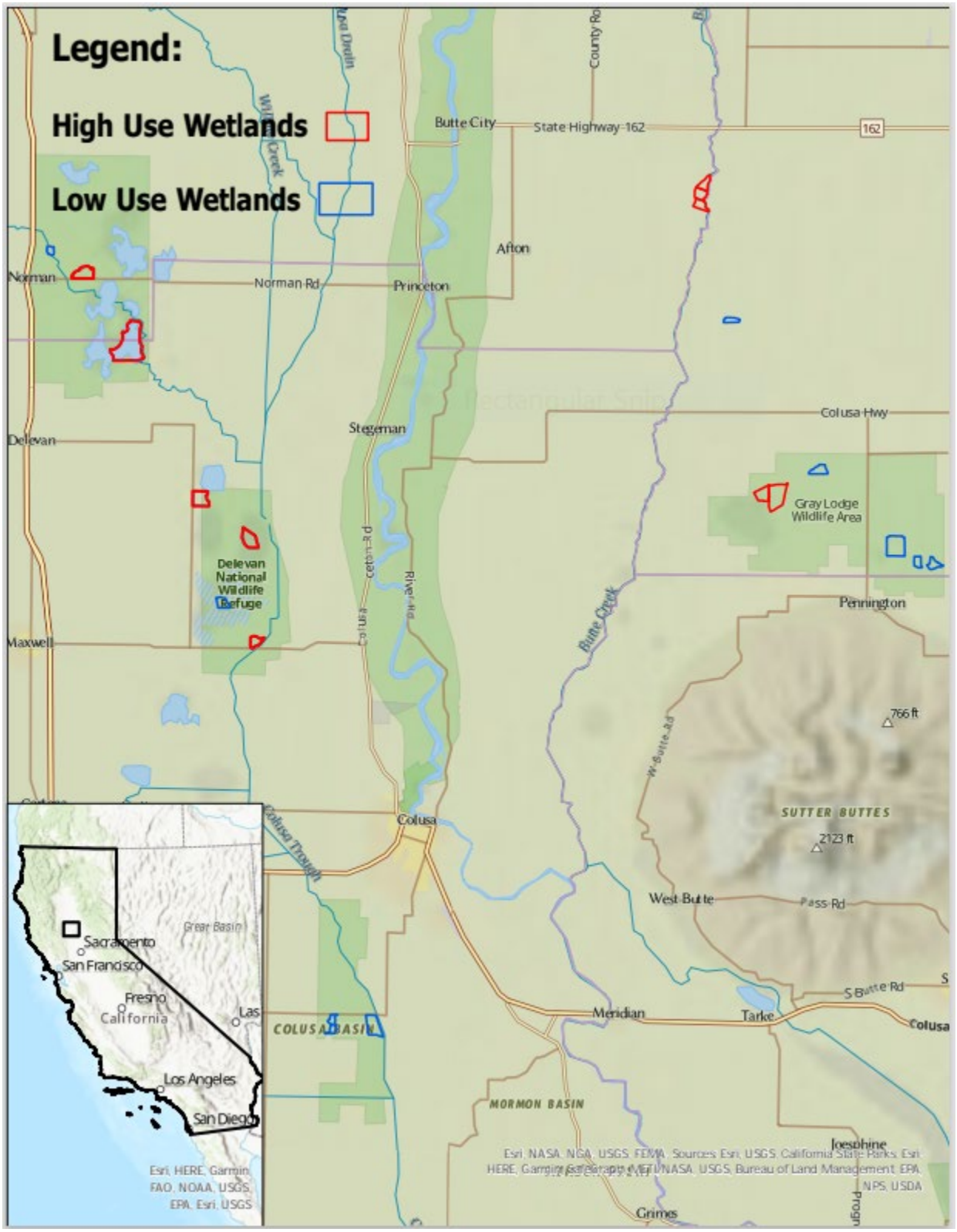
Legend:
High Use Wetlands
Low Use Wetlands
Butte City
State Highway 162
162
Afton
Norman
Norman Rd
Princeton
Stegeman
Delevan
Colusa Hwy
Gray Lodge Wildlife Area
Delevan National Wildlife Refuge
Pennington
Maxwell
766 ft
SUTTER BUTTES
2123 ft
Colusa
West Butte
Pass Rd
Meridian
Tarke
Colusa
COLUSA BASIN
MORMON BASIN
Grimes
Josephine
Sacramento
San Francisco
Fresno
California
Las
Los Angeles
San Diego
Great Basin
Esri, HERE, Garmin, FAO, NOAA, USGS, EPA, Esri, USGS
Esri, NASA, NGA, USGS, FEMA, Sources: Esri, USGS, California State Parks, Esri, HERE, Garmin, FAO, NOAA, USGS, Bureau of Land Management, EPA, NPS, USDA

Table 1. Sample collection over three sampling periods.

Sampling Period	Basin	Filtered Water Samples	Surface Water Samples	Soil Samples	Avian Swabs
Summer 2021	Sacramento Basin	50	10	50	182
	Butte Basin	50	10	50	--
Winter 2022	Sacramento Basin	50	10	50	80
	Butte Basin	50	10	50	80
Winter 2023	Sacramento Basin	50	10	50	80
	Butte Basin	50	10	50	80

Figure 2a. Prevalence of AIV in water samples according to wetland usage.

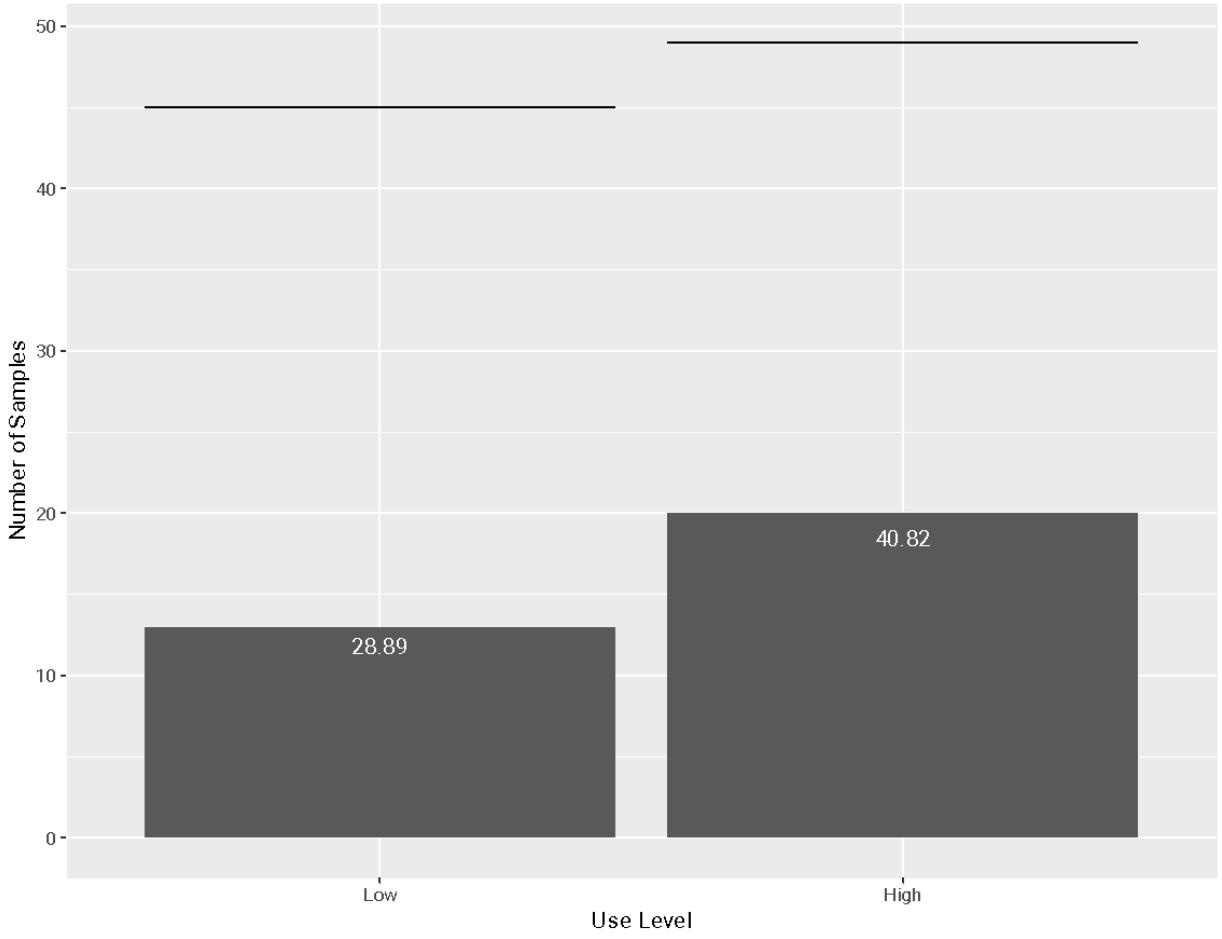

Figure 2b. Prevalence of AIV in sediment samples according to wetland usage.

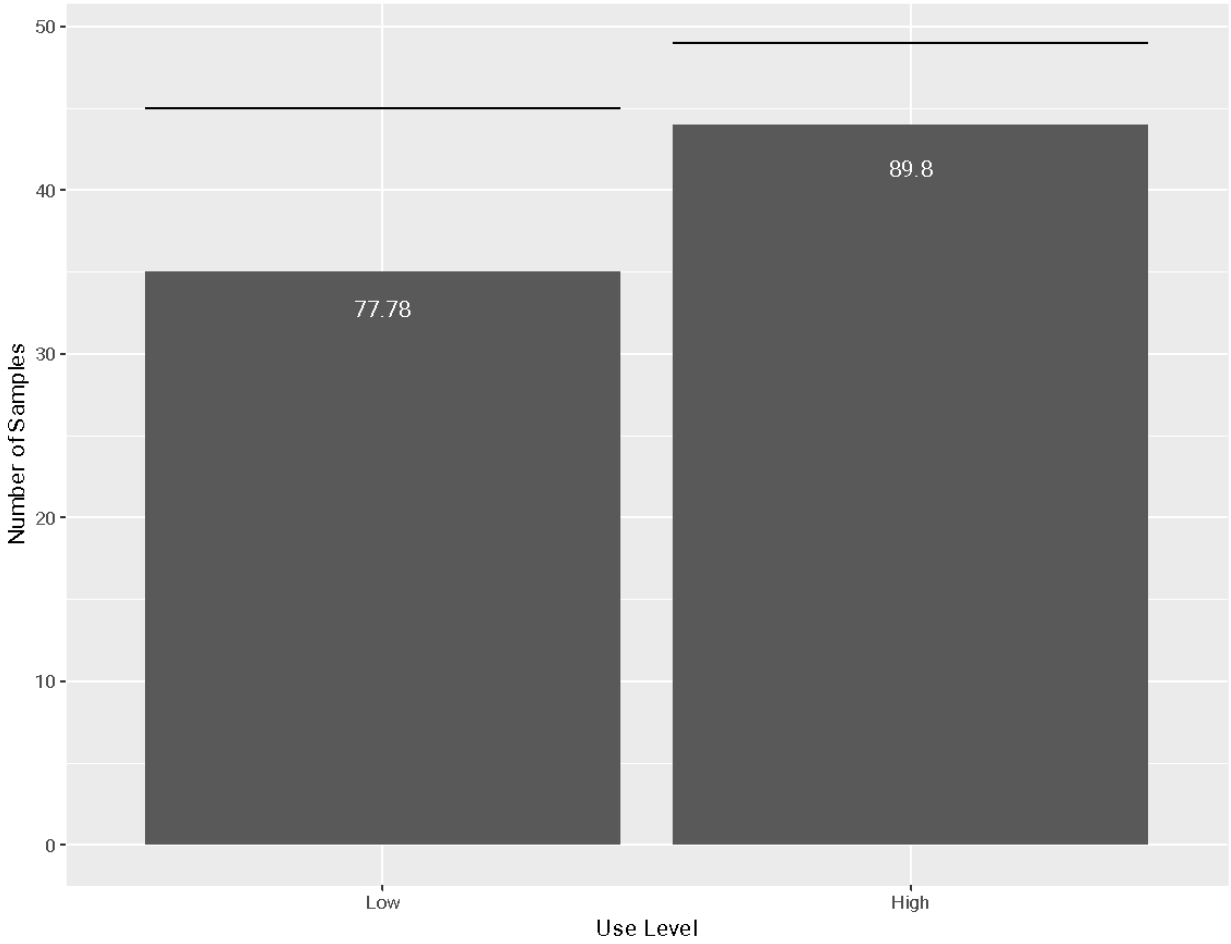
50
40
30
20
10
0
Number of Samples
77.78
89.8
Low
High
Use Level

Table 2. Environmental conditions of positive samples.

Sample ID	Use Level	Filtered	Date	Time	pH	Temperature (C)	Conductivity (uS/cm)	Dissolved Oxygen (mg/L)
C24.7A	Low	Y	8/27/2021	0858	7.54	21.5	709	0.78
C24.7E	Low	Y	8/27/2021	1000	9.19	21.1	1354	0.74
C24.8D	Low	Y	8/27/2021	0908	7.59	21.7	758	0.29
C27.1A	Low	Y	8/20/2021	0921	8.76	19.9	610	5.17
C27.1B	Low	Y	8/20/2021	0925	7.99	21	674	2.65
C27.1C	Low	Y	8/20/2021	0948	7.7	21.3	792	2.07
C27.1D	Low	Y	8/20/2021	0950	7.8	21	780	2.46
D27.5D	Low	Y	8/18/2021	1212	7.26	22.3	483.4	0.69
D44.2B	High	Y	8/13/2021	0935	7.59	22.8	555	4.95
D44.2C	High	Y	8/13/2021	1004	9.32	24	503	1.06
D6A	High	Y	8/18/2021	0917	7.55	20.8	551	2.62
D6B	High	Y	8/18/2021	0949	7.72	20.4	545	4.09
D6C	High	Y	8/18/2021	1013	7.49	19.9	478.5	4.56
D6D	High	Y	8/18/2021	1038	7.38	20	476.9	1.98
D6E	High	Y	8/18/2021	1053	7.47	21.5	538	2.2
G18A	High	Y	9/3/2021	0956	7.41	21.1	365.6	4.94
G18B	High	Y	9/3/2021	1007	7.38	21.8	493.9	1.08
G18SW	High	N	9/3/2021	1026	7.5	22.5	517	2.74
G36E	Low	Y	9/3/2021	0933	7.18	20.3	392.2	0.11
G54SW	Low	N	9/8/2021	1021	6.69	23.1	288	0.05
H204A.A	High	Y	8/31/2021	1020	7.25	23.8	369.7	0.68
H204A.B	High	Y	8/31/2021	1027	7.16	23.2	358.9	1.15
H204A.D	High	Y	8/31/2021	1047	7.19	22.7	299.8	1.54
H204A.E	High	Y	8/31/2021	1056	7.22	24	329.4	2.29
H204B.C	High	Y	8/25/2021	1000	7.16	24.8	287.5	0.86
H204C.B	High	Y	8/31/2021	0933	7.13	24.7	296.3	1.58
H204C.SW	High	N	8/31/2021	1006	7.06	22.8	301.9	1.59
S16.2A	Low	Y	8/20/2021	1057	7.03	20.5	169.6	1.3
S16.2D	Low	Y	8/20/2021	1139	7.08	21.2	229.9	0.68
S16.2E	Low	Y	8/20/2021	1148	7	21.3	164	1.9
S4B	High	Y	8/13/2021	1420	6.99	25.4	401.7	4.82
S4D	High	Y	8/13/2021	1450	8.59	29.4	1244	6.03
S4E	High	Y	8/13/2021	1458	8.6	25.7	1617	2.27

EVALUATION OF FIELD PERFORMANCE OF BROILER CHICKENS UNDER DIFFERENT COCCIDIOSIS CONTROL PROGRAMS

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SUMMARY

In the broiler industry, anticoccidial drugs and live coccidia vaccines are widely used approaches for coccidiosis control in chickens. Anticoccidial drugs, which are either chemical compounds or ionophores, are often applied in various combinations to improve the effectiveness of the treatment. On the other hand, a live coccidia vaccine is a prophylactic approach that can induce long-lasting protective immunity in the chickens and is a sustainable option for long-term use in the field without the development of resistance. To understand how different coccidiosis control programs affect the overall performance of broilers in the field, one year of the US broiler field performance data (from 2020 January through 2020 December) was obtained and multiple performance indexes such as feed conversion ratio and flock mortality were analyzed in this study.

INTRODUCTION

Coccidiosis, an intestinal parasitic disease caused by *Eimeria*, poses great economic significance in the poultry industry. *Eimeria* is transmitted via the fecal-oral route. Once sporulated oocysts are ingested by the host, several rounds of asexual and sexual reproduction cycles are followed in the intestine (1). During the reproduction cycles, *Eimeria* penetrates the intestinal epithelial cells and damages the physical integrity of the gut (2). The extended epithelial damage over a short period leads to diarrhea, dehydration, weight loss, and other clinical symptoms and young animals recovered from coccidiosis may show their growth compensated (2). Both clinical and subclinical coccidiosis are responsible for economic loss by increasing mortality and reducing feed efficiency. The economical loss due to loss of profits and cost of prevention and intervention has been calculated to exceed USD 3 billion annually (2, 3). To prevent coccidiosis, two approaches are widely used in the poultry industry: anticoccidial medications as feed additives, and vaccines. Since the first paper demonstrated the control effect of coccidiosis by including a low level of sulfa quinoxaline in the feed of chicken in the 1940s, various anticoccidial drugs have been used in feed as a prophylactic treatment of coccidiosis (4). These anticoccidial medications can be classified into either chemical compounds or ionophores (considered antibiotics in the U.S.), and each has a different level of effectiveness toward each *Eimeria* spp. as well as a different mode of action. To improve the effectiveness of the anticoccidial activity, wide varieties of chemical and ionophores combinations are used. Due to the ubiquitous and continuous usage of anticoccidial medications in poultry farms, varying levels of anticoccidial resistance are found in the field (5). As political pressure and social, and medical concerns regarding the overuse of antibiotics has increased, production programs such as No Antibiotics Ever (NAE) or Antibiotic-Free (ABF) are more widely used. Unlike coccidiostatic or coccidiocidal effects by anticoccidial medication, a vaccine provides an immunological approach. For Eimeria vaccination, live un-attenuated or attenuated strains are usually used. *Eimeria* infection induces long-lasting protective immunity in the host, but the immunity is very specific to that particular *Eimeria* species. Therefore, the vaccines are prepared by mixing multiple species of *Eimeria*. In general, coccidia vaccines are applied via a spray vaccine machine mixed with water, or gel-type media and chicks ingest the sporulated oocysts in the vaccine via pecking and preening behavior. The controlled level of exposure to the oocyst allows the bird to build immunity without experiencing clinical coccidiosis. For the development of uniform immunity in the flock, adequate oocyst reproduction in the host and reinfection from the litter is required [6] and multiple factors such as chick density, litter condition, and humidity play an important role in this oocyst recycling system. Good poultry farm management is a key factor for the successful vaccination against *Eimeria*. Many studies on coccidiosis controls have been conducted using battery cages or floor pens, but the environment does not truly reflect the field situation, and the implications of the study findings are often limited. To understand how different coccidia control programs affect the performance outcomes of chickens in the real world, we obtained aggregated de-identified broiler performance data from January through December 2020 from

an industry partner. This blinded data set provided multiple performance indexes such as feed conversion ratio and flock mortality of farms with various coccidiosis control programs.

MATERIALS AND METHODS

Data. The production information was provided as blinded data. Any identifiable information such as a producer name, location, bird volume, weight volume, or plant number was removed before the data was received. Data were available from Jan 2020 through December 2020. The database contained poultry production information collected monthly from an individual reporting unit: the processing plant. In the US, vertically integrated poultry producers represent more than 90% of poultry production, and the organization of a boiler integrator consists of a production complex, which comprises a hatchery, a feed mill, a group of poultry growing farms, and a processing plant. To simplify the analysis, a bio-shuttle program (vaccination followed by in-feed anticoccidial medication) and a program using both chemical compounds and ionophores were excluded. Additionally, for anticoccidial medication, only compounds in the starter and grower feeds were considered when referring to the coccidiosis control program.

There are multiple different products available within the categories of chemical compounds and ionophores and vaccines. For this study, the names of each product used in the farm were not provided, thus the data were sorted based on the general category: chemical, ionophores, or vaccines. Coccidiosis control programs were classified as follows: ionohophore compounds in both the starter and the grower feed (ION-only), chemical compounds in both the starter and the grower feed (CHEM-only), and vaccination at the hatchery and no other anticoccidial medication in the starter and grower feed (VAC-only). Maxiban® (Elanco), a combination of an ionophore (narasin) and a chemical compound (nicarbazin), was classified as an ionophore in this study.

Performance report included calorie conversion (adjusted to 6.7 lbs. weight), weekly and total percentage mortality. In the dataset, the market bird sizes were ranging from 3.6 lb through > 8.5 lb. For the study, the birds were divided into three weight ranges: less than 4.4 lbs. (< 4.4 lbs.), From 4.4 lbs. through 6.8 lbs. (4.4 – 6.8 lbs.), and more than 6.8 lbs. (> 6.8 lbs.).

Statistical analysis. Data were analyzed by Kruskal Wallis nonparametric analysis of variance and tested by Dunn all pairs for joint ranks Post Hoc method using JMP software (version 16.2.0, SAS institute Inc. Cary, NC, 1989-2021). p values < 0.01 were considered statically significant.

RESULTS AND DISCUSSION

The total number of plants included in this study was 1404, including 6,505,509,494 heads of broiler. Based on USDA 2020 report (7), this number represented 70.5% of the 9,222,100,000 heads of US broiler produced in the US during 2020. Among 1404 plants in the study, 289 plants produced broilers in the weight range of < 4.4 lbs. and of these, 69.6% of the plants were on CHEM-only program, followed by 20.8% for ION-only and 9.7% VAC-only programs. 482 plants produced broilers in the 4.4-6.8 lbs. range, and 73% of the birds were treated with the CHEM-only program, followed by 22.2% of the VAC-only program and 4.8% of the ION-only program. For larger birds that weighed more than 6.8 lbs. (> 6.8 lbs.), among a total of 633 plants, 47.2% were treated with the CHEM-only program, and 23.7 % and 29.1% for both the ION-only and VAC-only programs respectively (Figure 1).

The weekly mortality and the total mortality of the birds under each program were compared. The weekly mortalities of each weight range were combined for each week (Figure 2). In figure 2, the blue line indicates the weekly average mortality of birds on the CHEM-only program, the red line represents the ION-only program, and the green line represents the VAC-only program. From 1st week through 7th week, except the 3rd week, the VAC-only program showed a significantly lower mortality than the CHEM-only program. While weekly mortalities for both the ION-only and the VAC-only programs were comparable until 5th week, the ION-only program shows an increase in mortality at 6th and 7th weeks. During the 8th and 9th weeks, there was no statistical difference among the three groups. The total cumulative mortality (%) during the life of flocks for each weight range was compared between the coccidiosis control programs (Figure 3). While there was no statistical difference in total mortality between the three treatment groups for the birds weighing < 4.4 lbs., the VAC-only program had a significantly lower mortality compared to the CHEM-only program for birds of 4.4- 6.8 lbs. and > 6.5 lbs.

The total live production cost (cents/live lb.) is the cost associated with raising the chicken from eggs to the processing age. The cost includes chick cost (including hatchery vaccination cost), house cost, feed ingredient cost (including in-feed medications), field vaccine and field medication cost, condemn cost, and other management associated costs (catch & haul, overhead, etc). The total live production cost of plants under the CHEM-only program had a mean of 38.36 ± 2.43 cents/live lb. and was significantly higher than those of the ION-only program (37.22 ±

2.4), and the VAC-only program (37.20 ± 2.18). There was no statistical difference between the total live production of the ION-only and the VAC-only programs.

Adjusted calorie conversion (adjusted to 6.7 lbs. weight) is a measure of feed efficiency (Figure 4). When compared within the same weight range, all treatment groups in both small (3.6-4.4 lbs.) and large (>6.5 lbs.) birds showed no difference. However, the plants under the VAC-only program showed a significantly lower adjusted calorie conversion compared to the CHEM-only program for the birds weighing between 4.4 – 6.8 lbs.

In summary, the US broiler production data collected from January through December 2020 were analyzed to compare the performances of birds under different coccidiosis control programs. The results showed that the birds treated with the CHEM-only program had statistically higher total live production cost, and weekly and total percentage mortality compared to the birds treated with the ION-only or the VAC-only program. When considering the feed efficiency, represented by adjusted calorie conversion, the data showed that the VAC-only program was not only comparable to the CHEM-only and the ION-only programs but also had a significantly better feed efficiency in the mid-size birds weighing between 4.4-6.8 lbs. The overall results indicate that the performance of the birds treated with chemicals in the starter and the grower feed did not surpass the performance of the vaccinated birds. The US industry data of the 1 year provided a chance to look at the impact that a coccidiosis control program can have on broilers in the field. However, the data had its intrinsic limiting factors to consider. Although coccidiosis can impact poultry performance, multiple factors such as quality of feed, management, disease status, and environmental condition have a huge influence on the poultry production outcomes. As the provided blind data did not include any of this information, the analysis was done without considering other influencing factors. In addition, there was no detailed coccidiosis control program information provided, therefore the difference existing between the different products within the same category of the anticoccidial medications (chemical, and ionophores) as well as vaccine manufacturers could not be considered in this analysis. When considering the impact of the coccidiosis control program on the bird performance, the previous flock's history including its coccidiosis control program is important as consecutive use of the same anticoccidial medication can increase resistance in the field. Ideally, following overall performance through multiple production cycles in a specific farm could provide a better understanding of the impact of the coccidiosis control program on performance. Even with its limitation, the analysis of the US field data allowed us to compare the overall performance difference of birds treated with chemicals, ionophores, and vaccines.

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Figure 1. Percentage of coccidiosis program of each broiler weight range.

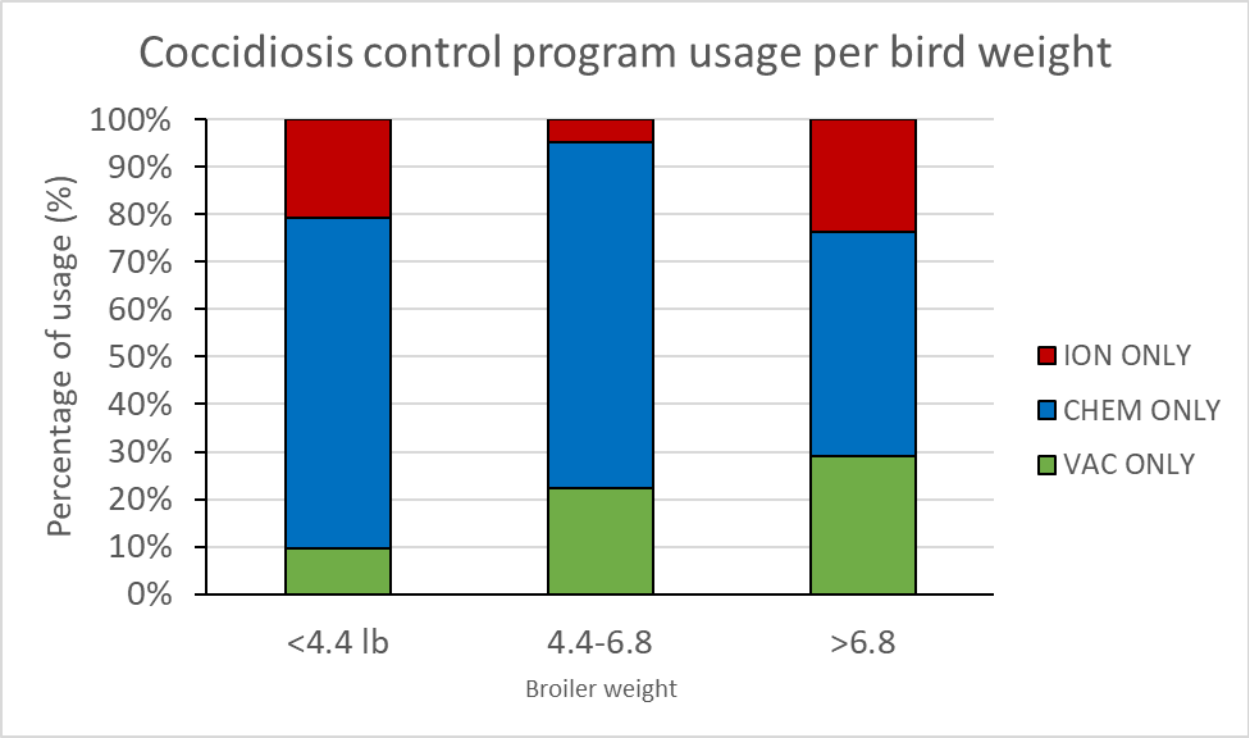
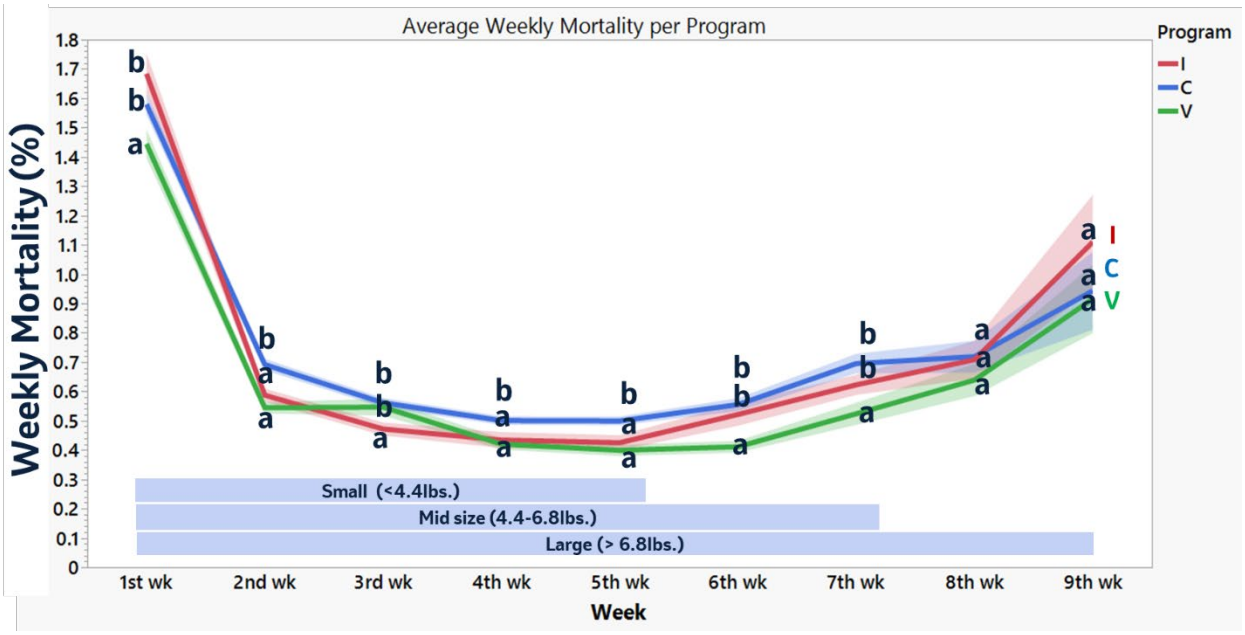
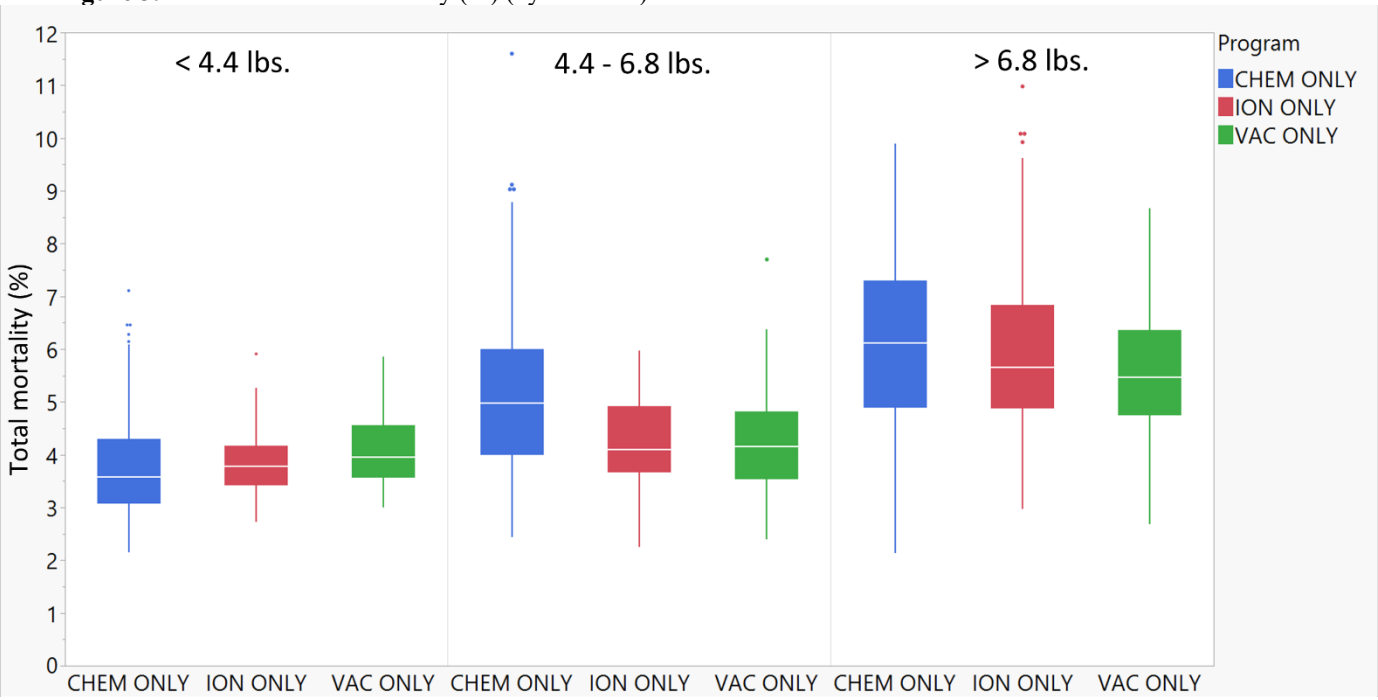


Figure 2. Weekly average percentage mortality (all weight combined).



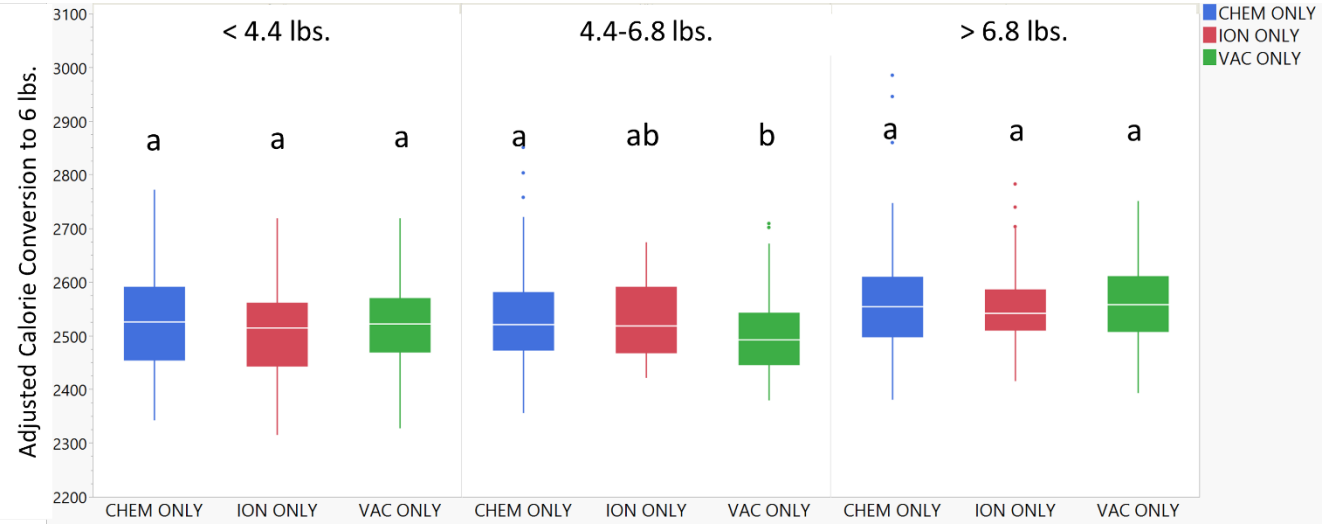
The error band (shades of the lines) were constructed using a 95% confidence internal of the mean.
Different letters indicate statistical differences at p <0.01

Figure 3. Total cumulative mortality (%) (by bird size).



Different letters indicate statistical differences at p <0.01

Figure 4. Adjusted calorie conversion (adjusted to 6.7 lb wt).



Different letters indicate statistical differences at p <0.01

EVALUATION OF TWO DIFFERENT DAY-OF-AGE IBV SPRAY VACCINATION PROGRAMS ON EARLY PROTECTION AGAINST DMV/1639 IN LEGHORN PULLETS

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SUMMARY

A typical IBV post-hatch vaccination program with a Mass-type vaccine via spray was compared to vaccination with a Mass+Ark combo to evaluate their protection against a DMV/1639 challenge at 21 days (Study 1) and 10 days (Study 2). Protection was measured by doing PCR of internal organs, such as trachea, kidney, and ovary, and by histopathological evaluation of tracheas. The Mass+Ark combo provided better protection of internal organs and tracheal pathology than the Mass-only program at a 21-day challenge, and comparable protection at a 10-day challenge. These studies showed that a Mass+Ark combo vaccine at day-of-age in pullets can provide comparable, if not, better protection against early DMV/1639 challenges, and it could lay out a broader IBV baseline to build upon with the booster vaccinations.

INTRODUCTION

Infectious bronchitis is one of the most important and challenging diseases for the poultry industry. Layers are usually affected during egg production, causing respiratory signs, egg production drops, and eggshell quality issues. Early protection through multiple vaccinations of pullets with different IBV strains is critical for the control of this disease throughout the life of the birds. The DMV/1639 strain of IBV has been particularly challenging in the last few years since early infections can lead to a condition called False Layer Syndrome (FLS), and certain traditional vaccination programs can fail in protecting the birds against a challenge with this strain later in their life. Therefore, it is now a common practice to apply monovalent IBV vaccines, usually of the Mass serotype, via spray cabinet at day-of-age in the hatchery to prevent the presentation of FLS. The objective of these studies was to compare a monovalent (Mass only) against a bivalent (Mass+Ark) post-hatch IBV program to evaluate their protection to a DMV/1639 challenge at 10 and 21 days.

MATERIALS AND METHODS

For each study, 480 healthy SPF Leghorn chicks were randomly assigned to three treatments: Non vaccinated, Cloned Mass, and Mass+Ark combo. 32 birds were placed per isolator, with three isolators per treatment for the challenged and 2 isolators per treatment for the non-challenged. Birds were vaccinated at day-of-age via spray cabinet using 14mL/100 chicks. The design of each study is shown in Table 1.

The evaluation of protection was performed as follows:

- PCR: A specific probe for DMV/1639 was used. Any result with a CT ≥35 was considered protected.
    1. Study 1: Trachea and kidney
    2. Study 2: Trachea, kidney, and ovary
- Tracheal thickness: A cutoff was determined from the mean mucosal thickness of the non-challenged birds ± 2 SD. Any value above the cutoff was considered non-protected.
- Tracheal pathology: Any bird with a score ≥3 for gland loss, cilia loss, or lymphoid inflammation was considered non-protected.

All hypotheses were conducted at the $p \leq 0.05$ level of significance with the Shaffer simulated method used to adjust for multiple comparisons.

RESULTS

Study 1 – 21d Challenge

- Both vaccines had a 100% of positive takes (CT≤35) on Day 7.

- The Mass+Ark combo showed significant better protection of the trachea and numerical better protection of the kidney than the Cloned Mass (Figure 1). The mean CT of the Mass+Ark combo was 38.9 for trachea and 39.1 for kidney, while the mean CT for the Cloned Mass was 36.6 and 38.2 respectively. The mean CT of the positive controls was 25.3 for trachea and 33.5 for kidney.
- The Mass+Ark combo provided significant better protection against tracheal pathology (94% vs. 67%) and a numerical better protection from tracheal thickness (97% vs. 81%) than the Cloned Mass.

Study 2 – 10d Challenge

- Both vaccines had a 100% of positive takes (CT≤35) on Day 7.
- The Mass+Ark combo provided a numerical better protection of the trachea and ovary than the Cloned Mass. The protection of the kidney was equally effective (Figure 2). The mean CT of the Mass+Ark combo was 39.5 for trachea, 40 for kidney, and 40 for ovary, while the mean CT for the Cloned Mass was 38.3, 40, and 37.9 respectively. The mean CT of the positive controls was 23.3 for trachea, 31.6 for kidney, and 25.6 for ovary.
- The Mass+Ark combo showed a numerical better protection against tracheal lesions than the Cloned Mass (88% vs. 77%) and had a slightly lower protection against tracheal thickness (64% vs. 67%).

DISCUSSION AND CONCLUSIONS

Arkansas-type vaccines have been instrumental in the layer industry to control and broaden the protection against IBV field challenges and virtually all the pullet vaccination programs include this serotype. However, to our knowledge, its effectiveness as a post-hatch primer IBV vaccination together with a Mass serotype has not been evaluated. These studies demonstrated that the Mass+Ark combo at day-of-age provides comparable, if not, better protection of internal organs against an early DMV/1639 challenge than a Mass vaccination alone. Another potential benefit of the bivalent program is that it establishes a broader priming baseline to build the booster vaccinations onto, likely improving the overall immunity against IBV challenges throughout the bird's life.

Table 1. Design of each study.

Action	Study 1	Study 2
Vaccine Takes	Days 7 and 21	Days 7 and 10
Challenge - 4.3 EID^{50}/bird, intraocular	Day 21	Day 10
Termination and evaluation of protection	Day 27	Day 16

Figure 1. % Protection of Internal Organs vs. DMV/1639 Challenge at 21 days (CT≥35)

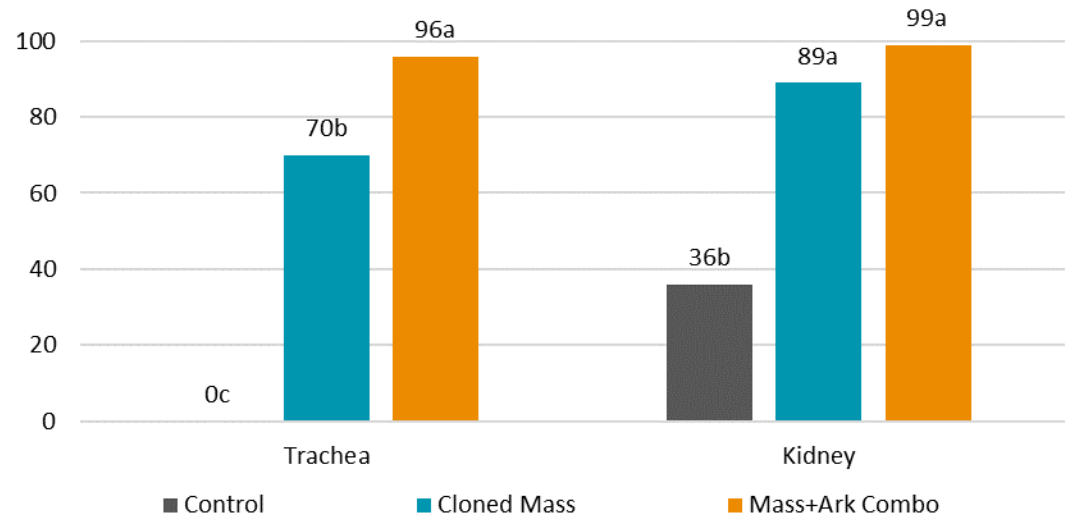
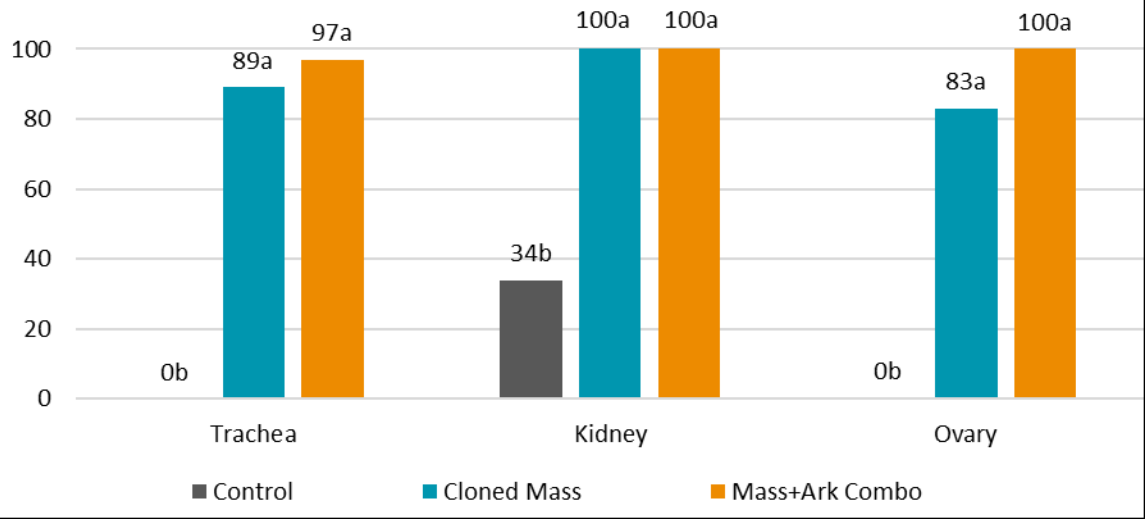


Figure 2. % Protection of Internal Organs vs. DMV/1639 Challenge at 10 days (CT≥35)



RELEVANCE OF AVIAN HEPATITIS E IN LAYER FARMS IN THE UNITED STATES

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INTRODUCTION

Avian hepatitis E virus (AHEV) is a non-enveloped, single-stranded RNA virus that belongs to the genus Orthohepevirus. It is the responsible agent of hepatitis-splenomegaly syndrome (HSS) in layers and is clinically characterized by increased mortality and decreased egg production. However, AHEV has also been reported in chickens without HSS symptoms (1, 2). At present, 4 genotypes have been identified worldwide. Genotype 1 includes AHEV isolated from chickens in Australia (3), genotype 2 from the USA (4), genotype 3 from Europe (5) and China (6), and genotype 4 is a new genotype from Hungary (7). According to serological databases HEV is widespread in chicken flocks with seropositive rates of approximately 71% in the United States, 90% in Spain and 57% in Korea (8, 9, 10). Currently, not much information is available on the epidemiology and impacts of HEV in laying hens in the United States. Therefore, it is important to look for epidemiological patterns of the virus in laying hens to provide important information concerning the presence and epidemiology of HEV. The objective of this study was to assess the presence of AHEV or their antibodies by collecting tissues and blood from farms with clinical signs using molecular and antibody detection and electron microscopy.

MATERIAL AND METHODS

Clinical specimens. Spleen and liver tissues were collected from 30-layer type chickens of 35 weeks of age. Serum samples were collected from 79 birds. All serum samples and tissues were kept frozen at -20°C until processing.

ELISA to detect anti-avian HEV antibodies in chicken sera. Chicken serum was tested for anti-AHEV IgY antibodies by enzyme linked immunosorbent assay (ELISA) using the Big Liver and Spleen Disease Antibody test kit (BLS CK 131, BioChek, Berkshire, United Kingdom) according to the manufacturer's protocol.

Electron microscopy negative contrast. Seven livers and three spleens were tested by this diagnostic technique. This method is recommended for screening of viruses allowing their identification up to family level. The samples were diluted 1:10 in cold phosphate-buffered saline. The diluted suspension was centrifuged at a temperature of 48°C at 1300g for 10 min; this process was repeated with the cleared supernatant. The supernatant was then ultracentrifuged for 15 min (91,124g at 130 kPa) on carbon coated Pioloform copper grids. Hydrophilicity of the carbon surface of the grids was achieved by UV irradiation and immersion in Alcian blue. Negative staining was performed using 1% aqueous uranyl acetate and 1% aqueous phosphotungstic acid. The samples were finally analyzed in a Zeiss 906 at 80 kV.

RESULTS AND DISCUSSION

Gerbert et al reported in United States that the seropositive rate of antiavian HEV IgY antibodies was 44.8% in sexually mature layers at various stages of egg production and that all farms studied were seropositive for anti-avian HEV IgY antibodies with a detection rate ranging from 20% to 82% per farm. The results of this study evidenced the presence of antibodies to avian HEV in 14 out of 79 serum samples (17%). A quick look into our California diagnostic laboratory system did not find HEV indications during the last five years.

Serological assays alone are inadequate in screening for acute avian HEV infection. Viremia and fecal virus shedding occur in infected birds much earlier than the appearance of avian HEV IgG antibodies and thus, seronegative birds could still be infected with avian HEV (13), in the present study tissue samples were processed by negative staining electron microscopy, and they were negative. EM is a methodology that is not sensitive, reason why we are currently working on establishing molecular detection tests and histopathology techniques to demonstrate the presence of the virus or the effects that the virus poses in tissues. The results of the current testing will be shared at the meeting.

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TARGETED LEARNING MODULES FOR THE POULTRY INDUSTRY ON HIGHLY PATHOGENIC AVIAN INFLUENZA

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SUMMARY

Avian influenza is a recurring disease that has devastating impacts on the poultry industry. Poultry organizations struggle to handle outbreak situations due to changes with employees, prevention measures, and the speed with which decisions need to be made. Our goal is to create a course with five modules, each composed of 15 to 20-minute classes that will provide organizational leaders with fundamental knowledge of Avian Influenze (AI). The first four modules will be done independently for individuals to reflect on their own departments and needs during an AI outbreak. The fifth module is an in-person session for major decision makers to come together to build their strategic plans. From this course, organizations will learn how to pair AI information with their niche-based departmental expertise through self-reflection questionnaires and group activities to improve the speed of decision making, communication among stakeholders, AI mitigation strategies, and allocation of financial and human resources.

MATERIALS AND METHODS

The five modules are created to assist poultry companies to prepare for an avian influenza outbreak. The first module discusses the basics of AI, how it spreads, the difference between low pathogenic avian influenza and highly pathogenic avian influenza, and how to determine the risk of infection to their companies. The second module covers what a farm's designation means during an outbreak, permitted movements, and the secure poultry supply plan. The third module talks about the emotional stress an outbreak can cause on everyone within an organization. The module contains techniques on how to identify signs of emotional stress or burnout, how to build an environment of connection, and how to proactively build relationships. The fourth module gives organizations tools on how to understand their informational needs during an outbreak. An organization will learn how to find trustworthy sources, how to use their uniqueness to their advantage to stop or prevent an outbreak, and how to create a message that will unite the entire their organization.

The last module, the strategic planning, brings all the major decision makers in a company together to build a plan on how to prepare for an avian influenza outbreak. Organizations will build complete a table top exercise to gain familiarity with the type of decisions that need to be made during an AI outbreak. During this table top exercise organizations will build a communication plan that identifies various audiences and their concerns to proactively build messages to prepare for those concerns. At the end of the strategic planning, organizations will have a developed an organizational AI team, and departmental AI crisis teams. These AI crisis teams will identify who is in charge of making decisions and how the information will be passed through an organization.

RESULTS

Currently both layer and turkey companies are piloting through the course and providing feedback through an evaluation.

CONCLUSION

This course is an opportunity for organizations to unite, prepare, and understand what an Avian Influenza outbreak would mean for them. It gives organizations the basics of what avian influenza is, when regulatory oversite is involved, how to manage emotional strain placed on employees, identify communication needs, and put together organization and departmental AI crisis teams.

WHY DO CHICKENS DIE WITH HIGHLY PATHOGENIC (HP) AVIAN INFLUENZA AND DUCKS MOSTLY SURVIVE? AN OVERVIEW OF THE IMMUNE RESPONSES TO HP AVIAN INFLUENZA VIRUS

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SUMMARY

Infection of chickens with highly pathogenic avian influenza virus (AIV) causes 100% mortality within 48 hours post experimental infection, which contrasts to infection with low pathogenicity (lp) AIV. In the latter case, chickens produce neutralizing antibodies and recover from the infection proving that there is no inherent deficiency in mounting an effective immune response to AIV infection. In contrast with chickens, waterfowl can experience mortality but can also recover from infection with hp AIV. In this review, I analyze differences in immune responses between chickens and ducks after infection with lp and hp AIV. Hp AIV infects several cell types in chickens including endothelial cells lining the blood vessels, while the latter cells are not infected in ducks. In addition, hp AIV replicates in thrombocytes in chickens but not in ducks. Infection of thrombocytes causes release of pro-inflammatory cytokines, upregulation of genes attracting heterophils and forming microthrombi. The combination of damage to the endothelial cells, activation of thrombocytes and heterophils causes leakage of fluid resulting in rapid mortality.

INTRODUCTION

Hp AIV is currently causing mayor problems worldwide in commercial poultry and free-living birds. In 2020, a new reassortment strain emerged in Europe with a change from H5N8 to H5N1 with the N1 gene derived from an infection in a wild bird. This virus, commonly referred to as WB-H5N1, spread first through Europe to Africa and Asia. The virus was detected in the USA in January 2022 (1) and has since spread into South America with recently confirmed cases in poultry flocks and free-living birds from Colombia to Chile. Like many of the earlier H5N1 variants, mortality in turkeys and chickens occurs very rapidly after infection often with 100% per acute mortality. In contrast, infection in waterfowl may cause mortality typically starting around 5 to 7 days post infection (dpi). In this review, I will use selected references briefly describing the differences in lp and hp AIV infection and mortality between chickens and waterfowl with an emphasis on immune responses and pathogenesis in chickens, mallard ducks (*Anas platyrhynchos*) and the closely related Pekin ducks.

Immune responses to lp and hp AIV in chickens. Infection of chickens with lp AIV normally results in a mild infection of the respiratory and intestinal tract and birds mount an immune response leading to recovery from the infection (2). Cleavage of the hemagglutinin (HA) protein is essential for infection and in lp AIV infection the cleavage of HA depends on trypsin, which is the major reason the infection remains in the respiratory and intestinal tracts. Relatively few publications have addressed the innate immune responses in chickens to lp AIV. Cornelissen et al (3) infected chickens with H7N1 lp AIV and detected viral RNA in the lung as early as 0.8 dpi. The pathogen recognition receptors (PRR) toll-like receptor (TLR)7 and melanoma differentiation-associated protein (MDA)5 were strongly upregulated in the lungs at 0.8 dpi, which correlated with interferon (IFN)α and IFNβ mRNA upregulation. All chickens seroconverted at 7 dpi. The humoral immune response after infection with lp AIV consists of IgM antibodies, which are detected as early as 5 dpi, and are followed shortly afterwards by IgY and IgA (4). Virus-neutralizing (VN) antibodies are directed to the HA protein and to a lesser degree to the neuraminidase (NA) protein. In addition, it is assumed that cell-mediated immune (CMI) responses are also generated but there is little information on the importance of CMI responses for the recovery from infection or protection to reinfection (2, 4). Killed or recombinant vaccines produce VN antibodies protecting against lp- and hp AIV as long as the HA protein from the vaccine strain is similar to that of the challenge virus (5). In conclusion, chickens can mount innate and acquired immune responses to lp AIV in the apparent absence of retinoic acid-inducible gene-1(RIG-1), which is a key PRR involved in the innate immune of ducks to AIV infection.

Obviously, infection of chickens with hp AIV causing per acute mortality within 24 to 48 hours post infection (hpi) does not allow the development of an acquired immune response. Several researchers have noted that infection with hp AIV causes a rapid and often dramatic increase in cytokine mRNAs. For example, Karpala et al (6) using the hp AIV H5N1 strain VN/1203 found a dramatic increase of IFN mRNA and IL12 in the lung and to a lesser degree in the spleen. The mRNA levels for pro-inflammatory cytokines IL6, IFNα, IFNβ and IFNλ were significantly increased in the spleen and especially the lung but not in the brain. Th2 responses such as IL4, Il10 and GATA3 were not upregulated by infection with VN/1203. When VN/1203 was changed to lp virus, the levels of mRNAs for the pro-inflammatory cytokines were similar to the levels in uninfected control chickens. Using different hp H5N1 strains, Burggraaf et al (7) also reported a significant increase of IL6 in spleen and lung.

Immune responses to lp and hp AIV in ducks. Infection of mallard ducks or Pekin ducks with lp AIV causes only a mild infection in the intestinal tract without overt disease (8). Infection of mallards with hp AIV may cause mortality depending on the specific strain of hp AIV. For example, limited mortality occurred after infection with VN/1203 starting after 5 dpi (9) while infection with dkVT453 caused severe disease starting at 4 dpi with 100% mortality or euthanasia at 5 dpi [7]. Wang et al [10] infected mallards with two H5N6 strains JA1 and SH1. Infection with the former caused mortality in 4/5 ducks. The latter did not cause disease in ducks but killed all infected chickens within 3 dpi. In addition to the virus strain, the duck species may also influence the incidence of morbidity and mortality with mallards being more resistant than other dabbling ducks (8). The reasons for these differences to AIV infection among duck species have not been elucidated.

Most research on the immune responses to lp and hp AIV in Anseriformes has been conducted in mallards and Pekin ducks. Fleming-Canepa et al [11] compared the innate immune responses in Pekin ducks to hp VN1203 (H5N1), a naturally arising attenuated variant of VN/1203 with one mutation in the PA gene (T515A), lp BC500 (H5N2) and lp BC544 (H5N9). Virus replication of VN/1203 and T515A were similar in the lung at 1, 2 and 3 dpi, but the relative expressions of RIG-1 and IFNB mRNA were significantly higher at 1 dpi in the lung of ducks infected with VN/1203 than with T515A: 157- versus 47.3-fold and 178- versus 27-fold increases, respectively. Infection with BC500 or BC544 resulted in weak innate immune responses based on expression of RIG-1 and IFNB. Downstream interferon stimulated genes (ISG) were also expressed at a significantly higher level after infection with VN/1203 than with the three lp strains. Evseev and Magor (8) summarized the differences between hp and lp AIV infection in mallard ducks. Hp AIV replicates in first instance in the respiratory tract with virus shedding from the trachea and virus can spread to other tissues. Infection results in a very rapid induction of type I IFNs, ISGs, and pro-inflammatory cytokines with high expression of ISGs in infected tissues. In contrast, lp AIV replicates primarily in the epithelial cells of the intestinal tract resulting in fecal shedding. There is minimal induction of type I IFNs and pro-inflammatory cytokines and low expression of ISGs in infected tissues. Interestingly, Wang et al (10) found that the H5N6 JA1 strain caused mortality in ducks accompanied by high levels of inducible nitric oxide synthase (iNOS) expression in the brain. In general, high levels of pro-inflammatory cytokines in ducks are correlated with more severe disease in ducks with mallards the least susceptible to cytokine storms compared to other duck species (8).

Ducks develop acquired immune responses to AIV. Surviving ducks infected with H5N1 hp AIV will develop hemagglutination-inhibition (HI) antibodies (9). Yang et al (12) infected ruddy shelducks (*Tadorna ferruginea*) and chickens with H9N2 AIV by the intranasal route. HI antibodies developed in chickens and shelducks, albeit a few days later and with lower titers in the ducks than in the chickens. Vaccination programs using oil-emulsion inactivated vaccines are used to prevent clinical disease and to reduce virus shedding. The protection by these vaccines is mostly correlated with the development of HI antibodies but HI titers are general lower in ducks than in chickens (13). The lower HI titers may be caused by the presence of truncated IgY (IgYΔFc), which does not participate in the HI response. It is not clear if IgYΔFc can neutralize virus (14).

Differences in cell tropism of hp AIV between ducks and chickens. Endothelial cells are a major target cell for hp AIV infection in chickens in contrast to infection in mallard and Pekin ducks (15). Schat et al (9) infected chickens with hp reverse genetic-generated (rg)VN/1203 and with lp rgVN/1203 in which four basic amino acids in the hemagglutination cleavage site were deleted. The predilection for endothelial cells was associated with the hp virus, while the lp virus caused a limited infection. Based on the detection of viral antigens, infection with lp strain occurred mostly in neurons, single glial, fibroblast or epithelium cells in different organs and dermis but not in endothelial cells. In the same study, ducks infected with hp rgVN1203 were frequently positive for viral antigen in cells in the heart and brain but not in endothelial cells. Interestingly, in vitro infection of duck and chicken endothelial cell lines showed that both cell lines can be productively infected with hp AIV but are displaying different transcriptional responses to virus infection (16). Chicken endothelial cell lines had a stronger pro-inflammatory response to infection than the duck endothelial cell lines but this is not specific for hpAIV infection because stimulation with polyI:C had a similar effect (16). These results raise an interesting question: why are duck endothelial cells not infected in vivo? The importance of infection of the endothelial cells in chickens is reviewed in (15). Infection

of endothelial cells results in apoptosis and thus leakage of fluid causing edema. It has been hypothesized that damage to the endothelial cells may affect the innate immune responses. In addition, the damage activates the extrinsic coagulation pathway causing microthrombosis and thrombocytopenia.

In addition to the difference in infection of endothelial cells between ducks and chickens, Schat et al (9) found that hp rgVN/1203 replicates in thrombocytes of chickens within 12 hpi in contrast with infection of ducks. The lp rgVN/1203 did not infect thrombocytes in chickens or ducks. This finding was not completely new, because Sterz and Weiss (17, 18) had shown in 1974 that thrombocytes were able to phagocytose and replicate "fowl plague virus" in vivo and in vitro. The importance of infection of chicken thrombocytes with hp rgVN1203 was further examined by transcriptome analysis of thrombocytes harvested 18 hpi. Significant upregulation of several genes was noted: e.g., cell adhesion genes (SELE, VCAM1, and CD274), interferon stimulated genes (MX), Toll-like receptor signaling pathway (STAT1, TLR3) and genes involved in the RIG-1-like receptor signaling pathway (TRIM25, TMEM173 and IFH1) (9). The importance of these findings for the pathogenesis are discussed in the next section.

Thus far, the importance of thrombocytes for the pathogenesis of viral infections has not been examined in chickens in great detail. In a recent review, Astill et al (19) described the importance of thrombocytes for phagocytosis of pathogens and innate immune responses in addition to its role in coagulation. Moreover, thrombocytes contain preformed pro-inflammatory cytokines, which are released upon stimulation. St Paul et al (20) had shown that stimulation of chicken thrombocytes with LPS and CpG oligonucleotides induced strong pro-inflammatory responses with more than 100-fold increases in IL1β, IL6 and IL8 transcripts. Stimulation of chicken thrombocytes with LPS resulted in the release of functional IL6 (Schat, unpublished data 2010).

A proposed model to explain the rapid mortality in chickens infected with hpAIV. Clearly, there are at least three major differences between chickens and ducks infected with hp H5N1 AIV: 1) differences in the cytokine responses, 2) infection of endothelial cells in chickens but not in ducks, and 3) infection of thrombocytes in chickens but not in ducks. Infection of endothelial cells and thrombocytes were associated with hp H5N1 VN/1203 but these cells were not infected when the cleavage site was changed to the lp form of AIV. Based on these results and our work on the early pathogenesis of infection [6, 9], I propose the following model to explain the rapid mortality after infection with hp AIV (Fig.1). The timeline from infection to being moribund is depicted in A in black color. Virus replication (B in blue color) starts with cleavage of the virus by trypsin and initial replication probably in epithelial cells of the lung. Further replication is facilitated by cleavage of the HA by furins and at 12 hpi virus is replicating in endothelial cells and starting in different organs including thrombocytes. At 18 and 24 hpi, virus replication is rampant in spleen, lung, brain and thrombocytes. The consequences of the infection are summarized in C (brown color). Damage to the vascular endothelial cells causes fluid leakage and formation of microthrombi. Infection of thrombocytes disseminates virus to the different organs. The importance of the thrombocytes is depicted in D (red color). The thrombocytes contribute early to the proinflammatory cytokine storm by releasing preformed cytokines and upregulation of TLR3, MX and other genes adding to the cytokine storm and causing additional vascular damage. In addition, cell adhesion molecules such as SELE and VCAM1 are upregulated in the thrombocytes attracting heterophils and binding these to the microthrombi causing additional damage to the endothelial cells with subsequent fluid release. The fluid release leads to a shock-like syndrome depicted by the red arrow (E) and rapid mortality. The fact that duck thrombocytes and vascular endothelial cells are not infected prevents the rapid release of fluids leading to a slower disease process and a change to recover. It would be of interest to examine if thrombocytes are infected in some of the swan species experiencing rapid mortality in which endothelial cells are infected (e.g., black swans) (15).

CONCLUSIONS

The rapid mortality in chickens after infection with hp AIV has frequently been attributed to the cytokine storm, which differs to some degree from the cytokine storm in mallards and Pekin ducks. The differences in target cells for infection in chickens and ducks (vascular endothelial cells and thrombocytes) are probably more important to explain the differences in the pathogenesis. In general, the importance of thrombocytes in the pathogenesis of many diseases has not been studied in detail. As indicated by Astill et al [19] it will be important to include the role of these cells in future studies on the pathogenesis of many diseases in chickens.

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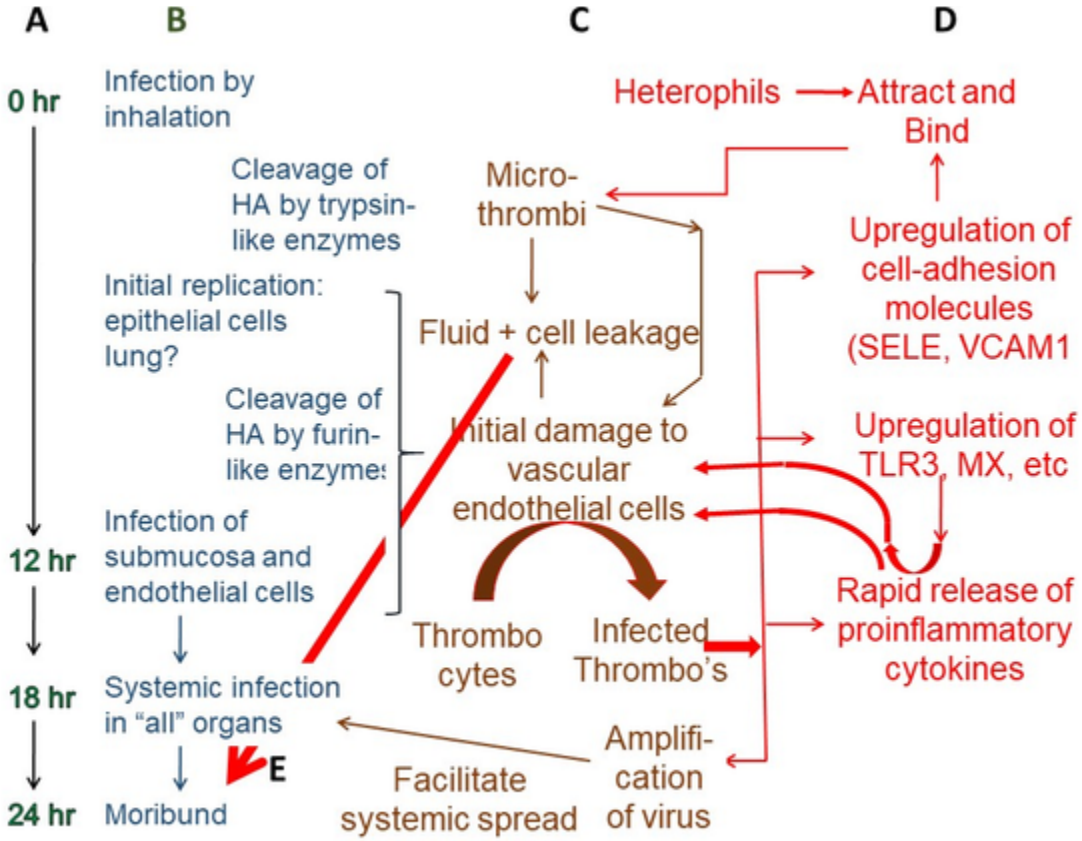
Figure.1. A proposed model to explain the rapid mortality in chickens after infection with highly pathogenic avian influenza virus. See text for explanation.



EFFECT OF A PROPRIETARY DIETARY ESSENTIAL OIL PRODUCT ON GROWTH PERFORMANCE, INTESTINAL LESIONS AND OOCYST COUNTS ON BROILERS DURING A MIXED *EIMERIA* CHALLENGE INFECTION

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SUMMARY

Historically, coccidiosis control programs have centered on chemical anticoccidials, ionophore antibiotics and vaccination regimens. As the global poultry industry moves away from the use of antibiotics, the opportunity to use ionophore antibiotics has declined. Some chemical or synthetic anticoccidials have also experienced pressure toward decreased usage. Alternative coccidia control strategies are being investigated. This report is a summary of two experiments conducted at Southern Poultry Feed and Research, Inc. (Athens, GA) to evaluate an essential oil (EO) product on growth performance, intestinal lesions, and excreta oocyst counts (OPG) after a challenge infection with *Eimeria coccidia*. In each experiment a statistically robust number of 1-d-old Cobb 500 broiler chicks were allocated to battery cages and segregated into three treatments. The three treatments were uninfected–unsupplemented control (UUC), infected–unsupplemented control (IUC), and infected + essential oil dietary supplementation (IEO). The experimental coccidia challenge occurred on d 14 where all birds except for UUC were orally gavaged with an appropriate amount of sporulated oocysts of *E. acervulina, E. maxima, and E. tenella*, respectively. On d 20, intestinal lesion scoring and oocyst enumeration of excreta contents occurred. Feed intake and body weights were measured on d 0, 14, and 20. Data were analyzed by ANOVA and means separated with Fisher's least significant difference at $P \leq 0.05$. In both studies, growth performance and coccidiosis lesion scores were significantly affected and coccidiosis lesion scores in the IUC group compared to the UUC group. Broilers fed the EO at either the 0.75 or 1 lb/ton level had significantly better FCR (feed conversion ratio) and minimized *Eimeria*-associated lesions. The results show that supplementation of EO in broiler diets improved FCR and minimized lesions and excreta oocyst counts during an *Eimeria* infection.

INTRODUCTION

Coccidiosis in the global commercial poultry production system continues to be a major economic as well as an animal welfare impact (1). The pressure to reduce ionophore antibiotic anticoccidials has heightened the need for alternative programs to manage coccidiosis. Chemical or synthetic anticoccidials also face pressure regarding usage reductions due to consumer pressure and the increased risk of resistance by field strains of coccidia against the anticoccidial agent.

The effect of natural products on reducing coccidiosis in poultry has been investigated for almost 100 years. Perhaps one of the first published records of reducing coccidiosis shows that feed containing 20% or 40% lactose or dry skim milk respectively, reduced the symptoms of then-named Eimeria avium (2). Additional studies have shown the anticoccidial effect of an assortment of phytogenic compounds such as saponins (3), yeast and minerals (4) as well as a mixture of oregano, saponins and inulin (5). Additional research into other alternative anticoccidial ingredients is continuing. This purpose of this report is to further investigate the effectiveness of a combination essential oil in an experimental multi-species coccidiosis challenge.

MATERIALS AND METHODS

Both trials were conducted at Southern Poultry Feed and Research, Inc. (Athens, GA). Both trials utilized a similar protocol except for the dosage of the treatment article. 144, 1-d-old Cobb 500 mixed-sex broiler chicks were randomly allocated to Petersime battery cages and segregated into three treatments, which resulted in six replications per treatment. The three treatments were uninfected–unsupplemented control (UUC), infected–unsupplemented control (IUC), and infected+EO (IEO) dietary supplement. The EO is a proprietary blend of essential oil ingredients;

(DeviSTAT Broiler NA, Devenish, Fairmont, MN.) The dosage of the EO in Experiment 1 was 0.75 lb./ton feed, and 1.00 lb./ton feed in Experiment 2. The experimental coccidial challenge occurred on d 14 where all birds except for PC were orally gavaged with 1 mL inoculum containing 100,000, 50,000, and 75,000 sporulated oocysts *of E. acervulina, E. maxima, and E. tenella*, respectively. The PC treatment birds received 1 mL saline. On d 20, intestinal lesion scores (ranging from 0 = none to 5 = severe) were evaluated from 5 randomly selected birds per cage using the Johnson and Reid lesion scoring scale. Excreta from each cage were collected for oocyst enumeration using the floatation method. Feed intake and body weight were measured on d 0, 14, and 20. Data were analyzed by ANOVA and means separated with Fisher's least significant difference at $P \leq 0.05$. Trending difference between results was established at $P \leq 0.10$.

RESULTS

In Trial 1 significant reductions in weight gain and adversely affected feed conversion rates (FCR) occurred in the IUC treatment group as well as the IEO groups when compared to the UUC treatment group (Table 1) between d 0-20. A significant increase in weight gain and improvement in FCR was observed in the IEO treatment group when compared to the IUC group during this same time period. These results correlate with the reductions in coccidia lesion scores in the IEO when compared to the IUC group. Additionally, a significantly lower level of total fecal oocyst levels was noted in the group fed the 0.75 lb./ton treatment article. The increase in weight gain in the IEO compared to the IUC group was 0.027kg between d 0-20. The improvement in FCR during the similar time frame was 0.11.
In Trial 2, during the d 0-20 timeframe comparison, significant reductions in weight gain and adversely affected feed conversion rates (FCR) occurred in the IUC treatment group as well as the IEO groups when compared to the UUC treatment group (Table 2). During this time period no significant increase in weight gain nor improvement in FCR was observed in the IEO treatment group when compared to the IUC group. Yet a numerical improvement is noted. These results correlate with the reductions in coccidia lesion scores in the IEO when compared to the IUC group. Additionally, a significantly lower level of total fecal oocyst levels was noted in the group fed the 1.00 lb./ton treatment article. The increase in weight gain in the IEO compared to the IUC group was 0.024kg between d 0-20. The improvement in FCR during the similar time frame was 0.12.

DISCUSSION

Both trials showed a reduction in weight gain and an adverse effect on feed conversion rates when the treatment groups were challenged with the mixed coccidia infection. Additionally, either significant or numerical improvements in these variables were observed with the addition of the EO treatment article, when compared to the IUC treatment group.

The improvements in performance variables directly correspond with the reductions in coccidia lesion scores and excreta oocyst counts between the IEO and the IUC treatment groups. Interestingly, numerically similar reductions in coccidia lesion scores were observed between Experiment 1 and Experiment 2 even though the amount of treatment article was increased by 33% (0.75 lb./ton to 1.00 lb./ton) between Experiment 1 and 2 respectively. Additionally, statistical significance between the treatment groups when comparing the performance variables between the two experiments did vary. The reduction in average coccidia lesion score was similar between the experiments. The reduction in this variable between Experiments 1 and 2 were 0.54 and 0.82 respectively.

It is postulated that the reason for the difference in statistical significance in performance variable results between the two experiments may be associated with the coccidiosis challenge. Based on the oocyst counts, the IUC oocyst count in Experiment 1 was 621,900/g excreta, compared to the same treatment group in Experiment 2 with an oocyst count of 244,762/g excreta. The reduction in oocyst shedding in Experiment 1 with the addition of the EO was 406,830/g excreta. Whereas the reduction in Experiment 2 was 154,460. It could be reasoned that this reduction in oocyst production is an indication of less replication of the parasite in Experiment 2 and ultimately less adverse effect on nutrient absorption. This would correspond to a difference in the reproductive capabilities of the selected coccidia strains used in the trials, instead of the effectiveness of the treatment EO.

Both experiments indicate that the EO treatment article at either the 0.75 lb./ton and the 1.0 lb./ton rate did reduce coccidia lesions, oocyst production and ultimately improve production variables when compared to no treatment. The two experiments do indicate the importance of assessing a multitude of variables when a challenge model is included in the protocol. It is noted that these experiments were battery studies, yet they do illustrate that the EO treatment article is a viable supplement when a strong coccidia challenge is present in broilers.

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Table 1. Results of Experiment 1: Effects of a mixed challenge infection of E. acervulina, E. maxima, and E. tenella on growth performance of broiler chickens when comparing three treatment groups.

	Uninfected-Unsupplemented Control (UUC)	Infected-Unsupplemented Control (IUC)	Infected-Supplemented (IEO)	SEM	P-value
	No Challenge	Challenge1	Challenge1 Essential Oil 0.75 lb./ton		
Weight Gain, kg/bird					
Day 0 – 14	0.320	0.311	0.318	0.015	0.69
Day 14 – 20	0.203 a	0.069 b	0.089 b	0.014	< 0.0001
Day 0 – 20	0.523 a	0.380 b	0.407 c	0.016	0.0351
Feed Conversion					
Day 0 – 14	0.969	0.970	0.957	0.034	0.97
Day 14 – 20	1.700 a	7.122 b	3.978 c	0.988	0.031
Day 0 – 20	1.372 a	1.765 b	1.654 c	0.042	0.039
Lesion Scores					
Eimeria acervulina	0 a	2.77 b	2.30 b	0.097	0.03
Eimeria maxima	0 a	1.88 b	1.33 c	0.135	0.01
Eimeria tenella	0 a	2.63 b	2.03 c	0.142	< 0.0001
Average of EA, EM, ET	0 a	2.43 b	1.89 c	0.078	< 0.0001
Oocyst Counts, n/gram					
Total of EA, EM, ET	0 a	621,900 b	215,070 c	60,922	< 0.0001

abMeans lacking a common superscript letter in a row differ ($P \leq 0.05$).

1All birds received 1 mL of oral inoculum containing a mixture of Eimeria acervulina (100,000 oocysts), Eimeria maxima (50,000 oocysts), and Eimeria tenella (75,000 oocysts) on d 14.

Table 2. Results of Experiment 2: Effects of a mixed challenge infection of E. acervulina, E. maxima, and E. tenella on growth performance of broiler chickens when comparing three treatment groups.

	Uninfected-Unsupplemented Control (UUC)	Infected-Unsupplemented Control (IUC)	Infected-Supplemented (IEO)	SEM	P-value
	No Challenge	Challenge1	Challenge1 Essential Oil 1.00 lb./ton		
Weight Gain, kg/bird					
Day 0 – 14	0.256	0.281	0.275	0.0186	0.2187
Day 14 – 20	0.254 a	0.134 b	0.164 b	0.0117	< 0.0001
Day 0 – 20	0.510 a	0.415 b	0.439 b	0.0263	0.0351
Feed Conversion					
Day 0 – 14	1.083	1.134	1.113	0.0402	0.3922
Day 14 – 20	1.371 a	2.274 b	1.873 c	0.0513	< 0.0001
Day 0 – 20	1.314 a	1.624 b	1.498 b	0.0406	0.0003
Lesion Scores					
Eimeria acervulina	0 a	2.77 b	2.33 b	0.118	< 0.0001
Eimeria maxima	0 a	1.97 b	1.30 c	0.117	0.0073
Eimeria tenella	0 a	2.77 b	1.40 c	0.226	0.0032
Average of EA, EM, ET	0 a	2.50 b	1.68 c	0.115	0.0005
Oocyst Counts, n/gram					
Total of EA, EM, ET	0 a	244,762 b	90,302 c	19,999	< 0.0001

abcMeans lacking a common superscript letter in a row differ ($P \leq 0.05$).

1All birds received 1 mL of oral inoculum containing a mixture of Eimeria acervulina (100,000 oocysts), Eimeria maxima (50,000 oocysts), and Eimeria tenella (75,000 oocysts) on d 14.

CHARACTERIZATION OF AVIADENOVIRUSES FROM CLINICAL CASES OF INCLUSION BODY HEPATITIS

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ABSTRACT

Clinical cases of inclusion body hepatitis (IBH) have increased in broilers in the U.S. and elsewhere in recent years. IBH is typically caused by aviadenoviruses belonging to groups D and E. Aviadenoviruses are the causative agents for IBH and can be classified into five species, A-E, based on the sequence of their genome. There are 12 recognized several serotypes within the five species, 1-7, 8a, 8b and 11. Over the past seven years, Aviadenoviruses belonging to fowl adenoviruses (FAV) group E, FAdV8b are the most prevalent viruses isolated and/or detected from clinical case submissions to the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia. IBH is a vertically and horizontally transmitted disease primarily affecting meat type chickens. No commercial vaccines for IBH adenoviruses are licensed for use in the U.S. and Canada and therefore companies have relied on the ability to produce autogenous vaccines for use in their broiler breeder flocks. The use of vaccines can reduce vertical transmission and provide maternal antibodies to broilers. Diagnosis of disease is based on clinical signs and lesions, as well as, histopathology, virus isolation and/or PCR detection. Serotyping by virus neutralizations and nucleotide sequence of the L1 hypervariable loop of the hexon gene are utilized by diagnostic labs to characterize the viruses. The primary aim of this study was to perform a retrospective analysis of IBH clinical cases submitted to PDRC from 2017-2022. Virus isolation and/or PCR were utilized to confirm presence of aviadenovirus in IBH clinical cases. IBH was observed at a higher prevalence in broilers between the ages of 22-29 days of age. Aviadenoviruses belonging to FAV group E, FAdV8b were the most prevalent viruses isolated and/or detected during 2017-2022. In addition, IBH was observed at a higher prevalence in broilers between the ages of 22-29 days of age. A second aim of this study was to perform serology from serum submitted to PDRC for FAV group E (FAVE), FAdV 8b (8b) and 8a (8a) virus neutralizations and compare to an FAV8ab ELISA to determine if the FAVE8ab ELISA could be utilized to evaluate serotype specific antibodies in field serum from adenovirus vaccinated and unvaccinated chickens. The results of the serological evaluation will help determine if the FAVE8ab ELISA kit can be utilized to monitor autogenous vaccinated birds. Given the significant increase in clinical cases of IBH and the widespread use of adenoviruses in autogenous vaccines, companies need a reliable serological tool for monitoring their vaccinated flocks and current licensed FAV ELISAs are not specific for any one serotype and virus neutralization assays are time consuming and expensive.

EVALUATION AND COMPARISON OF TWO METHODS OF WING WEB VACCINATION

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SUMMARY

In this study, wing web vaccination via the dose-specific, manufacturer provided handheld prong was compared to vaccination via a commercially available two needle pox vaccination gun. This study evaluated the differences in vaccine contamination, wing tissue pathology, and cost between the two methods of wing web vaccine administration. To enumerate bacterial contamination, 0.15 mL samples were collected during vaccination using both methods and culture and sensitivity were performed. To assess tissue pathology, 10 live birds were vaccinated once in each wing with each method and histopathology was performed on each wing 7 days later. Bacterial contamination of inoculum and cumulative wing pathology were higher in birds vaccinated with the pox gun than those vaccinated with the handheld prongs.

INTRODUCTION

Wing web vaccination is common practice in poultry for inoculation against fowl pox and other common poultry diseases. To ensure proper dosing and precise skin puncture, a dose-specific handheld prong is included with commercial wing web vaccines for administration, but commercially available pox guns are occasionally used as an alternative delivery method.

When using a handheld prong applicator in practice, each vial of vaccine is discarded when it is empty or near empty and a new prong is obtained for the new vial of vaccine. This means that only one vaccine vial and prong applicator is used per 1000 doses.

The commercially available pox gun (ThaMa Vet Syringes) contains two 14 gauge needles and a glass reservoir into which vaccine is poured. In practice, when the glass reservoir is near empty, a new vial of vaccine is poured into the reservoir on top of previously mixed vaccine. When using multiple guns, vaccine crews routinely combine vaccine from reservoirs of two or more guns to decrease wastage. Anecdotally, the suggested usage of a single needle prior to replacement is 50,000 to 100,000 birds.

The aim of this study was to evaluate the differences in vaccine contamination, wing tissue pathology, and cost between the two methods of wing web vaccine administration.

MATERIALS AND METHODS

Vaccine contamination. To evaluate differences in vaccine contamination, vaccine samples were obtained during routine vaccination of breeder flocks at 11 weeks of age. Vaccine crews skilled in the use of each method of vaccination were utilized on two farms approximately 30 minutes apart. Samples from each method were obtained at similar times, placed in a cooler, and all samples were delivered to the laboratory at the same time. Each sample was collected using a BD ½ cc U 100 insulin syringe and sent to University of Arkansas Veterinary Diagnostic Laboratory for culture and sensitivity. Samples were collected in slightly different ways due to how the two delivery methods manage vaccine. Sampling was designed to reflect the practices actually used by vaccine crews skilled in each vaccination method. Both sampling methods were designed to capture data when the chance of bacterial contamination was high.

Handheld prong inoculator method: A 0.15 mL sample was collected from the vial immediately prior to discarding the vial with unused vaccine and stabber. In this group, 9 total samples were collected at three timepoints from three different people vaccinating.

Pox gun method: A 0.15 mL sample was collected from the reservoir immediately prior to adding more vaccine. Samples were taken from both guns utilized by the vaccination crew, one was marked and the other was unmarked.

Before breaks crews routinely combine the vaccine from both reservoirs. In this group, 6 total samples were collected at 6 different time points.

Live bird/histopathology. The field samples previously tested had been aliquoted and were re-suspended in fresh tryptic soy broth and grown for 24 hours at 37°C. The field samples with the highest cfu were utilized as the vaccine/inoculum for this experiment. The overnight growth of the field samples utilized was 5x108 cfu for both methods of vaccination used in this experiment.

The wings of 10 broilers breeders were vaccinated with each vaccination method: for each bird, the right wing was vaccinated using the handheld prong inoculator and the left wing was vaccinated using the pox gun. New needles were used for both the handheld prong inoculator and the pox gun for the vaccination of all ten birds. Birds were vaccinated in each wing at 40 days of age. At 47 days of age, the birds were euthanized, the wings were removed and fixed in neutral-buffered formalin for histopathology. Three right wings were unable to be evaluated due to torn wing bands interfering with vaccination site.

Tissues were processed into 5-micron-thickness H&E slides for histologic measurements. A central section through the thickest central part of each specimen was selected for histopathology. Actual treatments were not known by the pathologist during processing, pathology data collection, or reporting. Lesions were identified and scored as 0, within normal limits; 1, minimal severity, 2, mild; 3, moderate; 4, marked; and 5, severe. The sum of lesion scores for each wing provided a cumulative pathology score.

Cost comparison. The cost of each method of vaccination was evaluated by determining the costs associated with each method. This included cost of the vaccination device, cleaning and disinfection associated with each device, maintenance/replacement parts, labor, and any additional equipment required for either method. Cost comparisons were based on a complex with placement of 960,000 birds per year.

RESULTS

Vaccine contamination. In the handheld prong group, bacteria was cultured from six of the nine submitted samples. Two samples from the first timepoint and one sample from the third timepoint did not culture bacteria. The bacteria cultured from this group included Enterococcus sp., coagulase negative *Staphylococcus* spp., and *Bacillus* sp. Bacterial enumeration of each sample is reflected in Figure 1.

In the pox gun group, bacteria was cultured from all six samples. All samples cultured coagulase negative *Staphylococcus* spp. and *Bacillus* spp. and one sample also cultured *Proteus* sp. Bacterial enumeration of each sample is reflected in Figure 1.

Live bird/histopathology. The most common histologic lesion was subacute to chronic myositis seen as necrosis of minimal to moderate numbers of muscle fibers accompanied by cellular inflammation, edema, and fibrosis. Cellulitis, seen as inflammation of dermal soft tissue, occurred in two chickens with mild to moderate severity. Sample number 5 from the left wing contained a linear track of cellulitis, accompanied by fibrosis, and intralesional bacteria, mostly coccoid bacteria but possibly a few rods. This lesion was consistent with a septic needle tract. Dermal fibrosis was identified in 2 samples from the left wing. Cumulative mean skin pathology trended higher in the left-wing specimens. Cumulative mean muscle pathology trended higher in the left-wing specimens. Cumulative pathology for all lesion scores trended higher in the left-wing specimens. Figure 2 summarizes histopathology results.

Cost comparison. The cost of replacement needles for the pox gun is approximately $12 for one needle, $25 for two needles, and $120 for a set of 10 needles. A spare parts kit which includes replacement rubber stoppers in the guns costs approximately $12. The cost of the guns plus maintenance is $135/gun. With approximately 80 placements per year and four gun cleanings required per placement, 320 gun cleanings are required for the year. Paying workers $15/hour for those cleanings, that amounts to approximately $4800 per year for gun cleaning. The needles will need to be changed approximately six times in the four guns for a cost of approximately $600. This results in a total program cost of $5,940.

The cost of handheld prong vaccinators is covered by the vaccine manufacturer. Two vaccination tables to assist in holding vaccine incur a one-time cost of $500. The tables should be cleaned at each placement for a total of 160 table cleanings at $15/hour wages, the cost of table cleaning is $400. Total cost for this program is $900 the first year and $400 every year after.

There is an 84.8% savings the first year using handheld prong inoculators and 93.2% savings every year after.

DISCUSSION

This experiment demonstrates that use of a 2 needle pox gun during commercial vaccination of birds results in higher levels of vaccine contamination and greater tissue damage. Clearly the intent behind the design of the pox

vaccine guns is to increase ergonomics of pox vaccination and increase the speed of vaccination. This data, however, suggests that these benefits may come at the cost of bird welfare and effective vaccination. When evaluated separately, use of a pox gun may be faster than use of a handhold prong however, when considering the vaccination process at this particular complex as a whole, the administration of eye drop vaccinations is the rate limiting step.

It is well established that pain associated with needle injections increases as the diameter of the needle increases (1,2). Owen et al. has also demonstrated that repeated needle use results in increased force required to puncture the skin, which can be reasonably assumed to increase pain and distress associated with needle puncture. The pox gun needle gauge is larger than that of the handheld prongs and is replaced much less frequently than the prongs. Additionally, the mechanics of the pox gun allow for less awareness of the increasing force required for needle puncture as the needles dull. Coupled with the evidence of inflammation visible in tissues vaccinated using the pox gun, it is reasonable to conclude that the pain associated with vaccination using the pox gun is greater than that associated with vaccination using the handheld prong applicator (1).

Efficacy of vaccination using the pox gun can also be called into question. The manufacturer states that the pox gun has a volume of 5 mL and a capacity for more than 1000 shots using that volume, which would amount to 0.005 mL/dose. Most commercial pox vaccines are labeled at 0.01 mL/dose, which the handheld prong is designed to deliver. Therefore, the pox gun is only administering a half dose of pox vaccine. The design of the pox gun also makes it more difficult to visualize filling of the needle reservoirs with vaccine. As the glass reservoir of the gun empties, the angle and torque of the gun must be adjusted to ensure the needle reservoirs fill and thus prevent dry hits. Additionally, the evidence in this experiment creates a need to reexamine how takes are evaluated when vaccinating using a pox gun. Takes generally consist of evaluating for swelling and scab formation at the site of vaccination. Trauma, bacterial contamination, and subsequent inflammation and edema from the use of the pox gun may contribute to the size of a take in vaccinated birds.

While this experiment incorporates a relatively small sample size, it clearly demonstrates potential welfare and cost benefits from transitioning vaccination from the use of pox guns to the use of handheld prong inoculators.

CONCLUSION

This preliminary study suggests the use of handheld prong inoculators for wing web vaccination results in less vaccine contamination and tissue pathology than a 2 needle pox gun vaccinator. Bird welfare and vaccination costs benefit from the use of handheld prongs during vaccination.

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Figure 1. Bacterial enumeration (cfu/mL) of samples collected from vaccination of birds with handheld prongs (left) and pox gun (right).

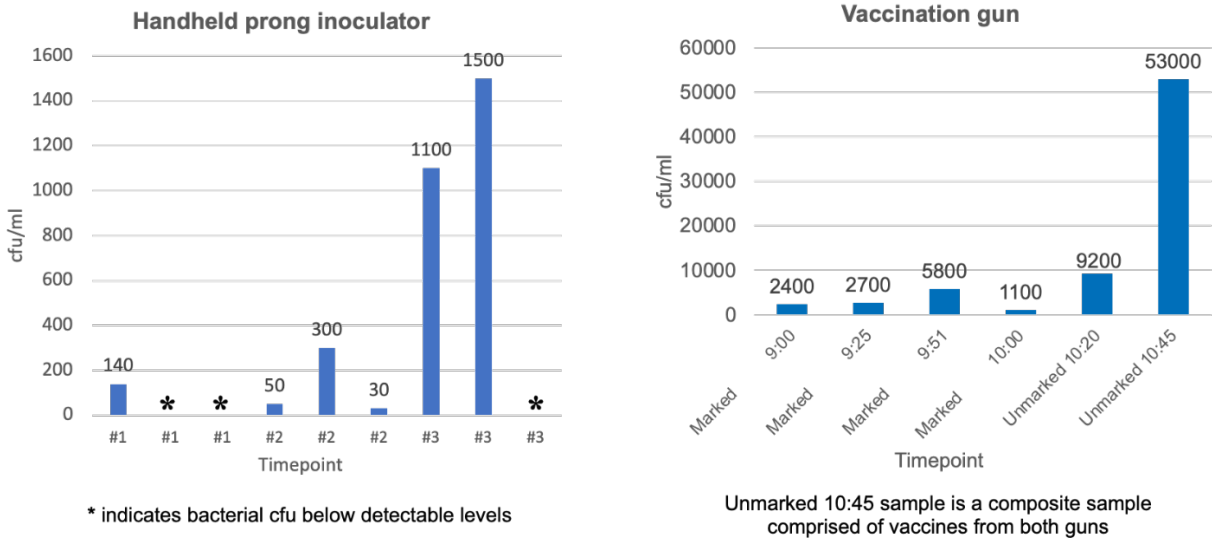
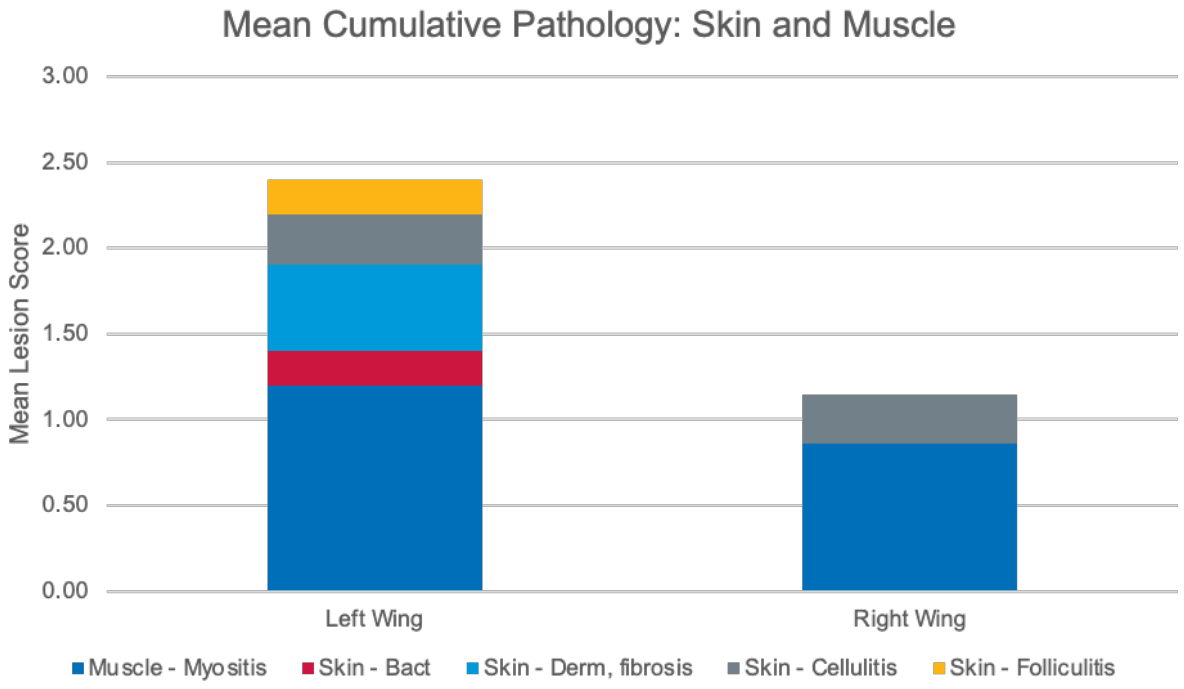


Figure 2. Cumulative pathology from pox gun vaccination (left wing) and handheld prong vaccination (right wing)



THE CHICKEN CLUSTER HOMOLOG OF IMMUNOGLOBULIN-LIKE RECEPTOR-B MOLECULES PLAY A SUPPRESSIVE ROLE DURING AVIAN INFLUENZA INFECTION *IN VITRO*

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SUMMARY

The full identity and roles of chicken cluster homolog of immunoglobulin-like receptor (CHIR) molecules during infection are not well established. We hypothesized that CHIR -B types, as classified by their intracellular inhibitory domain, suppress macrophage immune response against avian influenza infection. We searched online public repositories for chicken CHIR-encoding gene sequences and generated small interfering RNAs for gene-mediated degradation in in vitro macrophage culture prior to infection, co-culture with T lymphocytes, and detection of supernatant oxide species. Our results indicate that CHIR-B plays a role in suppressing nitrate production while restricting reactive oxygen species formation. This study adds to the importance of CHIR molecules in immunity and provides important implications for CHIR-based genetic selection in chickens.

INTRODUCTION

Immunoglobulin-like receptors are part of the immunoglobulin superfamily group of proteins that, in humans and mice, are known to have a direct role in response against extracellular pathogens. Depending on their structural characteristics, these transmembrane glycoprotein-situated immunoglobulin-like receptors can be activatory (-A) and or inhibitory (-B) in nature. These receptors' ligation leads to changes in cellular differentiation, either favoring or disfavoring inflammatory cascades for pathogen elimination.

Previous research indicates that some chicken cluster homologs of immunoglobulin-like receptor-A (CHIR-A) molecules bind with the hemagglutinin protein from the avian influenza virus (1). Secondly, the overexpression of what is now obsolete CHIR-A and -B sequence records suggests that these receptors likely interact with the major histocompatibility complex class I (MHC-I) molecule (2, 3). Therefore, these CHIRs can potentially regulate immune response through multiple means, via pathogen or MHC-I. However, further research is needed to pinpoint all gene sequences characteristic of CHIRs from online genetic repositories and classify whether they are, in fact, activatory and or inhibitory molecules. Proper annotation of these receptors on chromosome 31 of the chicken genome is important for clarification of their specific functions and involvement in broader biological processes. Without information on the molecular structure and empirical evidence for their role, it is difficult to convey the immunological action.

Therefore, this study aims to 1) define the full CHIR repertoire in the 2021 and 2022 chicken genome assemblies and 2) establish CHIRs' role in the immune response against viral infection, to understand their functional importance better. We expected to find at least 63 protein-encoding CHIR genes as previously recognized by fluorescence (4). Our annotation efforts not only led to more CHIR molecules being identified, but more than five named CHIRs needed corrections to their designated gene name. CHIR-B molecules, with the immunoreceptor tyrosine-based inhibitory motif, act functionally to suppress macrophage response against the avian influenza virus. In this study, we developed a bioinformatics pipeline and small interfering RNAs for gene-mediated degradation.

MATERIALS AND METHODS

Annotation. Protein and RefSeq Gene annotation of the bGalGal1.mat.broiler.GRCg7b and bGalGal1.pat.whiteleghornlayer.GRCg7w assemblies (Last modified in 2021,2022) were downloaded from the NCBI FTP site. InterProScan (v.5.52-86.0-v.5.56-89.0) and dependencies PFAM (v33.1), TMHMM (v2.0c), and PANTHER (v15.0) were used for protein classifications (5-8). SeqKit was used for retrieving ITAM ('Y.XXM'), ITIM ('[SIVL]XYXX[IVL]'), and transmembrane amino acids (9). MUSCLE (v3.8), IQ-TREE (v2.0, parameters –

maximum likelihood –bootstrap n=1000 replicates –substitution model AA, JTTmodel), and iTOL (v6.4.3) were used for constructing, modeling, and visualizing the phylogenetic relationships (10-12).

Generation of siRNA. Non-targeting and gene-targeting siRNA pools were designed and generated following the T7 RiboMAX Express RNAs System (Promega) with exceptions. In brief, four control sequences used elsewhere (13) were modified for length and flanking G and C, 5' and 3' ends before genome searching for complementarity in UCSC BLAT (Mar. 2018 GRCg6a) (14) and NCBI BLAST. Similarly, for the construct of gene-targeting siRNA pools, all potential 5'G(N)17C3' regions were identified from 26 representative CHIR-B genes. MCAST (v5.4.1, parameters –Motif p-value threshold <5e-11, –Output threshold E-value ≤10000) was used to observe for any overlap between CHIR -A/-B/-AB functional groups. As a result, a total of 12 consensus sequences were chosen to result in maximal CHIR-B group silencing. Preference was given to target sequences indicated to be most specific by NCBI BLAST and unlikely to form improper primer dimer pairing (Multiple Primer Analyzer, ThermoFisher Scientific). All DNA oligos were ordered from Europhins Genomics, and oligonucleotide pairs were annealed before pooling (v/v). For enhanced product stability and reduced immunogenicity, the 5-methyl-CTP and pseudo-UTP were substituted (1.25 mM final) in the RNA synthesis reaction, and dsRNA 5' ends were dephosphorylated using Antarctic phosphatase before silica column purification, as previous (15).

Control and NP_pcDNA-inoculated birds. Fertile eggs of different chicken strains (UCD331, UCD335, and WVU 1952) were kindly provided by Dr. Robert L. Taylor Jr. All protocols have been approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences. While the majority of the birds were not inoculated (control) and served as blood donors for our naive macrophages, 3 or more of the different UCD or WVU MHC-I genotypes (B2B2 or B19B19) were randomly selected for intramuscular inoculation at 10 and 70 days of age with 500 µg of the NP_pcDNA3.1. NP_pcDNA3.1 encodes the nucleoprotein (NP) of the H5N9 avian influenza virus, and this preparation has been used previously in our lab (16, 17). The inoculated birds served as blood T cell donors for co-culture, and T cells were collected at least 10 days after inoculation.

AIV stock. A low pathogenic avian influenza virus (AIV) (H5N9) was propagated in embryonic eggs (18) and tittered on primary and HD11 macrophages before use.

***In vitro* co-culture experiment.** Blood samples were collected by wing ulnar venipuncture into EDTA-coated tubes. The blood monocyte-derived macrophages (MDM) from our control birds were isolated using density gradient centrifugation and 5 days differentiation method (19) on 48-well or 96-well plates. For silencing, the MDMs from each donor were grown for 5 days under an elevated 8% CO2 level. On the 5th day of differentiation, we recovered T lymphocytes through differential monocyte attachment and B cell (Bu-1) depletion (Dynabeads). The MDMs were given a total of 500 ng of non-targeting (NTi) or CHIR-B (CHIR-Bi) siRNAs for 4 hours, media removed, infected with avian influenza virus (H5N9) for 1 hour, washed, and then subsequently co-cultured for 2 days with matched or mismatched MHC-I haplotype T cells from NP_pcDNA-inoculated donors. For the 2 days of co-culture, the cells were cultured under regular 5% CO2 levels. The cell supernatants were collected after co-culture for nitrate (Griess Assay) and hydrogen peroxide (H2O2) (ROS-Glo or Hydrogen Peroxide Assay kit from Cayman Chemical). The cell pellets were also collected after co-culture and are currently being analyzed for AIV viral load to associate with the cellular response. In addition, select samples will be sent for RNA sequencing to understand the precise downregulation and transcriptomic-wide effects of our siRNA pools.

Statistics. A linear regression or two-way repeated measures ANOVA with Fisher's Exact Post-hoc tests was used to analyze the effects. P value <0.05 was considered significant. In addition, post-hoc tests were performed to determine the trends.

RESULTS

To validate our siRNA silencing model for reductions in CHIR-B gene expression, we optimized cell differentiation at 8% CO2 (Figure 1-A). To validate our WVU 1952 animal model for differential nitrate responses, we included a B2B19 heterozygote. As described previously (18, 19), macrophages from B2B2 produced more nitrate, at 48 hours post-siRNA and -AIV application, than those from B19B19 (p=0.0038) (not shown). There was a linear trend of nitrate production, with the lowest nitrate production by the B19B19 macrophages, higher nitrate production by the B2B19 macrophages, and highest nitrate produced by the B2B2 macrophages ($F_{1,9}$=3.602, p=0.0902) (Figure 1-B). The highest nitrate responses out of the two haplotypes were MHC-I region B2 type.

Next, to exclude differences in the genetic background of the WVU 1952, we included the UCD congenic B2B2 and B19B19 chickens in the model. UCD-congenic B2B2 and B19B19 chickens differ simply in their MHC-I. The UCD lines produced significantly less nitrate than the WVU 1952 strain (p=0.0297); hence, the effects between the UCD and WVU lines were analyzed separately. In effect, CHIR-Bi marginally increased nitrate production in the UCD line (p=0.0690), in which CHIR-Bi nitrate was as much as 0.7770 µM lower that the NTi control and as much

as 13.28 μM higher than the NTi control (95% CI of difference) (Figure 1-C). While CHIR-Bi silencing did not increase nitrate in the WVU 1952 strain (p=0.4437), there was a numerical haplotype effect (p=0.0545) (Figure 1-D). Specifically, the mixed background B19B19 macrophage nitrate production was not different over silencing, but B2B2 macrophages produced significantly more nitrate than B19B19 macrophages when CHIR-Bi siRNA was applied (p=0.0344). Between UCD and WVU 1952 strains, CHIR-Bi tended to increase nitrate in the B2B2 (8.390 μM and 5.03 μM, respectively) haplotype more so than the B19B19 (4.115 μM and 0.183 μM, respectively) haplotype.

CHIR-Bi significantly decreased supernatant H2O2 species in the UCD (p=0.0393), and this change was numerically higher in B2B2 (-4020 RLU) than in B19B19 (-2312) (Figure 1-E). In the WVU 1952, the effect of silencing was significantly different between B2B2 and B19B19 (p=0.0131), where there was this significant decrease (-892 RFU) in H2O2 produced after CHIR-Bi silencing in the B19B19 (p=0.0454), and numerical increase (+739 RFU) in H2O2 produced after CHIR-Bi in the B2B2 (P=0.1118). B19B19 cells produced less or consumed more extracellular H2O2 than B2B2 cells after CHIR-Bi silencing (p=0.0218).

DISCUSSION

To better understand CHIR's role during infections, we designed our experiments to include different chicken strains and haplotypes. The WVU 1952 birds are selectively bred on their MHC-I type, whereas UCD birds were bred for genetic similarity apart from their MHC-I type. The WVU 1952 strain B2B2 and B19B19 birds have shown differential resistance and susceptibility, respectively, to a multitude of infections (20-23). Also, for the reason that immunoglobulin-like receptor binding with certain human leukocyte antigen class I molecules relate to increased viral load in humans (24), we distinguished CHIRs effects by haplotype.

We showed that CHIR-B silencing improves cellular nitrate release, which is congruent with its anti-inflammatory role in humans and mice (25, 26). However, the impact of CHIR-B silencing is not as robust in the B19B19. We speculate that it could be related to the high baseline expression of CHIR-B molecules and their signaling molecules. Data within our lab indicate that B19B19s, in comparison to B2B2s, have high basal CHIR gene expression, and their CHIR expression, in contrast to B2B2s, is reduced by interferon-gamma stimulation. Higher basal CHIR-B circuitry, such as the constitutive association of SH2-containing tyrosine phosphatase SHP-1 (27), in the B19B19, could prevent inductions of the immune response.

For the reasons that nitrate is 1) an accumulated product in our culture with an estimated relatively short half-life, 2) that nitrate was only detected from half of the birds, and 3) it may not be the only oxygen species used in defense against pathogens, we detected for changes in H2O2. Therefore, we expected extracellular H2O2 production for pathogen elimination. However, this was not a mechanism observed in our co-culture experiment. Rather, CHIR-B silencing augments the redox cascades differences. We speculate that reactive oxygen species such as H2O2 are produced when the mitochondria detect deleterious superoxide and are eliminated through superoxide dismutase and that H2O2 is consumed after reaction with metalloproteins in the Fenton reaction (28). Still, it is unclear from our culture if the lower H2O2 represents increased decomposition and altered cellular stress or if the lower H2O2 species comes from impaired redox signaling and superoxide dismutase metalloenzyme activity. Since the WVU B2B2 represents the immunocompetent type, background redox genes are implicated in disease resistance.

In conclusion, CHIR-Bs can functionally suppress macrophage response against the avian influenza virus. Further experiments are underway to ascertain the transcription regulators of CHIRs and pathways involved in CHIR signaling.

(The full-length article will be published in a journal to be determined later.)

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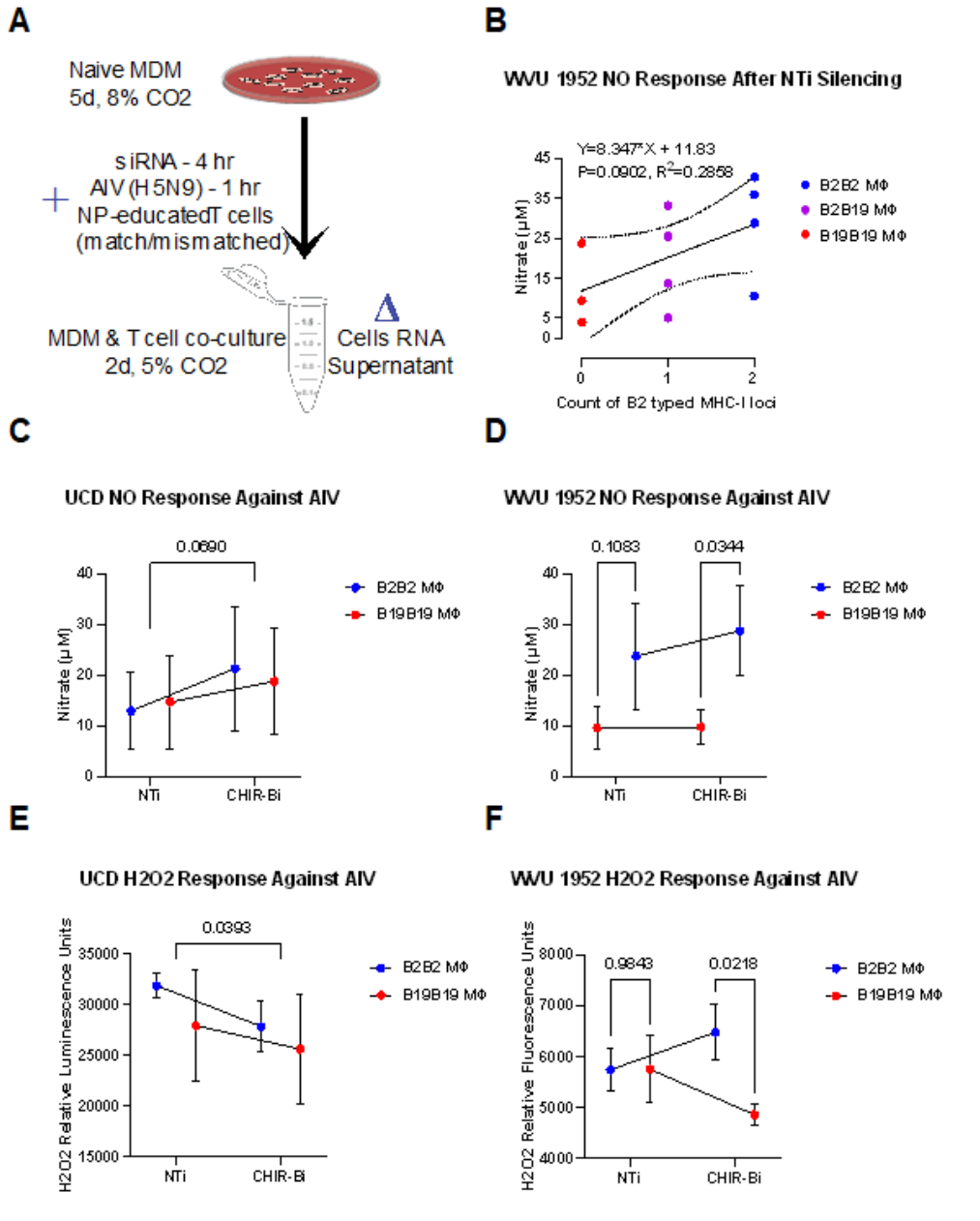
Figure 1. Effect of pooled non-targeting (NTi) control, or pooled CHIR-B (CHIR-Bi) mediated silencing (i), on MHC-I haplotype-specific immune response as measured by nitrate (NO) and hydrogen peroxide (H2O2). (A) An illustration of our in vitro study workflow. (B) NO response by WVU 1952 strain haplotypes after NTi. The line of best fit and individual biological replicates (n=3-4) are illustrated. (C) (D) NO response by UCD (n=3) and WVU 1952 (n=5-10) strain haplotypes, respectively. Mean and sem are illustrated. (E) (F) H2O2 response by UCD (n=5) and WVU 1952 (n=20-23) strain haplotypes, respectively. Mean and sem are illustrated. Only matched MHC-I co-culture responses are reported. P values identify a numerically different slope from zero, a significant effect of silencing, or pair-wise comparisons between haplotypes.



AVIAN INFLUENZA - KEY ASPECTS OF THE AVIAN HOST IMMUNE RESPONSE

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SUMMARY

Avian influenza (AI) is one the most pathogenic viruses in the poultry industry. There are a number of significant challenges this group of viruses presents to birds and mammals including zoonotic risk, barriers to international trade of live poultry and poultry products, as well as direct impacts on flock performance and mortality.

Avian influenza is one of the most studied viruses in the poultry industry, and yet there is much to learn, especially in terms of the avian host immune response. This talk will review unique aspects of the avian host immune response to AI including some areas of overlap between avian and mammalian systems.

DISCUSSION

Over the last several decades, researchers have improved our understanding of several aspects of AI including the makeup of surface proteins and how these proteins interact with host immune systems and receptors on the cellular surface. It is through this understanding that we have gained an understanding of how the virus adheres to the host cell, gains entrance to the cell, and how new virus particles are released into the extracellular environment.

AI is a member of the Orthomyxoviridae group of viruses, which are enveloped, segmented, single-stranded RNA viruses. There are three types of influenza viruses A, B, and C. Types B and C viruses only infect humans; type A viruses can infect a wide range of species including mammals and birds (1). Type A influenza can infect a wide range of birds including many types of domestic poultry; however, the normal host range includes primarily wild waterfowl and shorebirds. There have been numerous outbreaks of AI over the last several decades, many of which have been associated with contact between waterfowl and poultry, either directly or indirectly. While the mechanisms of viral transmission from primary sources to domestic poultry are often difficult to determine, transmission via direct bird to bird contact or indirect contact with virus-contaminated materials are considered likely.

Another important consideration is host specificity of the viruses which infect domestic poultry. Evidence indicates that natural interspecies transmission among poultry and other avian species has occurred. However, the extent to which AI is restricted by host specificity of a particular strain of virus is not clear. For example, some strains of AI that are capable of replication in chickens are restricted in their ability to replicate in ducks. Numerous studies over the years with isolates from outbreaks in domestic poultry have demonstrated the variation in host susceptibility. One could argue that a given isolate has unique characteristics of host range and capability of causing disease. Studies support the unpredictability of these viruses, and the complete host ecology of influenza is not known. Because of the complex interaction between wild birds, a complete understanding is unlikely (2).

In addition to the variation in host ecology, there is wide variation in avian host response to AI, from asymptomatic infections to high morbidity and mortality. Clinical signs include a range of respiratory clinical signs, production losses, and in some cases high mortality. One could argue that highly pathogenic strains of AI are some of the most devastating viruses the poultry industry faces. The standard method for classifying viruses as being either mildly pathogenic avian influenza (MPAI) or highly pathogenic avian influenza (HPAI) is the intravenous pathogenicity index, which states that if 6 or more 4–6-week-old SPF chickens die within 10 days of i.v. inoculation of 0.2 mL of a 1/10 dilution of infected allantoic fluid, the virus is considered HPAI. Most HPAI viruses fit into this category, however the definition has been expanded in recent years to include those viruses that have the genetic propensity to become HPAI based on the HA cleavage site.

Much has been learned about the differences between MPAI and HPAI viruses and their ability to cause disease including the differences in ability to replicate systemically or locally. HPAI viruses have the ability to replicate in a wide range of tissues due to the HA protein being able to be cleaved by endogenous proteases that are present in most cells of the bird. However, MPAI viruses are only cleaved by trypsin-like proteases primarily found in the enteric and respiratory systems (3). These aspects of tissue distribution between HPAI and MPAI viruses has been studied using immunohistochemistry techniques. These studies have demonstrated the presence of HPAI viruses in a range of tissues (via immunospecific staining for avian influenza viral proteins) including heart, lung, kidney, brain, and

pancreas. However, with MPAI viruses, the presence was infrequent and only in lung, trachea, and upper respiratory tract (4).

Measuring humoral immune response in poultry can be useful in determining presence or absence of infection, with the presence of IgM measurable as soon as 5 days post infection. Antibody that is produced is targeted against a range of viral proteins including the surface proteins, the internal proteins, and nonstructural proteins. Influenza has three types of surface proteins, Hemagglutinin (HA), Neuraminidase (NA), and M2. The HA and NA proteins are the primary means to classify AI, including pathogenicity. HPAI have thus far been associated with those of H5 or H7 HA subtypes, however the reasons as to why only these two HA types produce HPAI is not completely understood. The surface proteins are the only antigens capable of inducing neutralizing antibody and therefore a protective immune response (1).

The understanding of avian cellular immunology has greatly increased in recent years. Researchers have demonstrated this importance in a wide range of avian viruses, however more work is needed to understand the roles of cellular immunity and avian influenza. As mentioned previously, much of the work in mammals has been used as a guide for birds, without research being conducted within avian species.

Previous research on the immunomodulatory effects of influenza virus in mammals has been conducted using human influenza isolates and mammalian lymphocytes. Several studies have shown that influenza isolates were mitogenic for mouse B and T lymphocytes. Conversely, research conducted in other laboratories demonstrated that live influenza virus decreased the response of lymphocytes to T-cell mitogens and reduced antibody synthesis by B cells (5). It seems that the effect of influenza on mammalian lymphocytes needs more work. This being said, one could conclude that there has been less research with AI and avian lymphocytes as compared with mammalian studies. Holt et al studied the effect of avian influenza on chicken lymphocyte activation using Brucella abortus antigen (Ag)-primed lymphocytes, incubated with various doses of the T-cell mitogen concanavalin A (Con A) or Ag. AI enhanced the proliferative response and the enhancement decreased in a viral dose-dependent manner. The viral activity could be reduced by pre-treatment of the viral preparation with AI-specific antisera or prior adsorption of the AI with chicken erythrocytes. This study demonstrated that AI can interact with and modify the in vitro activity of chicken lymphocytes and may exert modulatory effects on the avian immune system.

Another interesting aspect of at least one isolate of HPAI (A/Turkey/Ontario/7732/66 H5N9) is that it has the capability to destroy lymphocytes in vitro and in vivo, independent of viral replication. While the mechanism of this destruction is not completely known, it may be due to replication in macrophages and the subsequent release of soluble factors that induce apoptosis, such as cytokines.

Studies in mammals of macrophages have demonstrated that these cells, which start as monocytes in peripheral blood, are scavenger cells that serve as the first line of defense against infectious organisms. Similarities between mammalian and avian macrophages certainly exist, including that these cells are important for nonspecific and specific immunity, and are important mediators of the inflammatory response. Studies in human and mouse macrophages have demonstrated that macrophages support influenza A replication, leading to macrophage cell death (6). An avian macrophage cell line (HD11) supported the replication of 13 HA subtypes [7], and splenic macrophages from chickens infected with the Ty/Ont strain were positive for virus, suggesting that these cells were infected in vivo (8). Macrophages are important in several ways including production of cytokines, phagocytosis of foreign proteins, elimination of cells undergoing apoptosis or necrosis, and internalizing/presenting antigens to T lymphocytes. These cells also produce free radicals and reactive oxygen species (ROS), which are important in terms of inflammation and microbiocidal activity (9, 10). It has been demonstrated in avian and mammalian macrophage studies that infection with influenza A viruses reduces functions including the respiratory burst, chemotactic response, and production of nitric oxide. A reduction in these functions may explain why there is an increased risk of secondary bacterial infections with influenza infections.

CONCLUSIONS

There is a wide variation in host ecology and host response to influenza A viruses. While we are beginning to understand more of the avian immunology of this important disease, much research is still needed. Researchers are understanding more about the complex interactions of these viruses with the host immune system, including lymphocytes and macrophages and where there is overlap of understanding between mammalian and avian systems.

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DEVELOPMENT AND DISTRIBUTION OF MAREK'S DISEASE VIRUS INDUCED TUMORS IN CHICKENS FOLLOWING EXPERIMENTAL INFECTION WITH THE VIRULENT FIELD STRAINS

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SUMMARY

Two pathogenic field strains of Marek's disease virus (JM and RB1/B) were tested in 1-day-old Specific Pathogen Free (SPF) chickens to compare their virulence, mortality, and tumor development in various organs/tissues following intraperitoneal inoculation. The RB1/B strain showed tumor development in various organs and tissues of experimentally infected chickens earlier than the JM strain. The results of the study indicated that in both strains, the kidneys were the most affected organ. Challenge strain RB1/B produced tumor associated death at a younger age versus the JM challenge strain. Overall, birds inoculated with the RB1/B strain showed higher tumor development in multiple organs than the JM strain. A comparison between the development and distribution of Marek's Disease Virus induced tumors between challenge strains is described.

INTRODUCTION

Marek's disease (MD), caused by the oncogenic lymphotropic Marek's disease virus (MDV), is a global concern for the poultry industry due to the unpredictable nature of the outbreaks in virtually all commercial flocks causing heavy annual economic losses to the poultry industry. The disease is clinically characterized by transient paralysis (2, 4, 8, 9, 12), early mortality syndrome (7, 11, 14), persistent neurological signs (7, 8, 12), gross ocular lesions and tumor formation in various tissues and organs (1, 4, 5, 7, 8, 11, 12, 14). The infection is caused by dust inhalation in the poultry house contaminated with MDV by the feather follicle epithelium shed from infected birds (5, 9). MDV is classified into three serotypes. Serotype 1 includes all the oncogenic strains of the virus along with attenuated strains (3, 7, 9) and is classified as the prototype virus of the avian disease (12). Serotype 2 consists of non-pathogenic strains of MDV isolated from clinically normal chickens (3, 7, 9, 12), and serotype 3 (Herpesvirus of turkey—HVT) is a non-oncogenic MDV-related virus isolated from turkeys (3, 7, 9). Serotype 1 is further divided into four pathotypes: mild (mMDV), virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV) (2, 7-9, 12, 14). The pathogenic strains of MDV are commonly used for vaccine evaluation by challenging vaccinated chickens through the sub-cutaneous or intraperitoneal routes. Due to variations in the pathotypes of the virus, choosing the right strain for vaccine evaluation is very important. Several challenge strains are now available for use in vaccine evaluation experiments such as JM (vMDV), RB1/B (vvMDV), MD5 (vvMDV) and GA (vMDV) (7, 9, 14). Different strains of MDV induce different lesions depending on both virus strain and genetic strain of the chicken (4, 13, 14). In this study, we focused on MDV challenge strains JM and RB1/B to compare their pathogenicity in the susceptible SPF chickens. The lesion development, prevalence and distribution caused by these two strains of MDV were compared.

MATERIALS AND METHODS

Experimental animals. One-day-old SPF White Leghorn chickens from Charles River were used in this study. The study consisted of 18 repetitive trials. In each trial, 20 – 25 chicks were used, and equal number of birds were kept as negative controls. A total 220 one-day-old chicks were used for challenging with the JM strain and 160 chicks were used for RB1/B challenge.

MDV challenge strains. Two pathogenic strains of MDV (JM and RB1/B) were used in this study. The challenge viruses were originally isolated from experimentally infected chickens by collecting their white blood cells and then giving a few passages in the chicken embryo fibroblast (CEF) cell monolayers. The CEF passaged viruses were cryopreserved in liquid nitrogen for future use. Prior to use, the cryopreserved viruses were thawed and resuspended in tryptose phosphate broth (TPB) in order to deliver 500 PFU of the virus in 0.5 mL inoculum per bird. Both challenge strains were processed following the same procedure.

Method of challenge. Each chick was inoculated with 0.5 mL of the diluted virus intraperitoneally using a 21-gauge, 1-inch needle and 1 mL syringe.

Post challenge observations and necropsy. All challenged and unchallenged control birds were observed 6 to 8 weeks for mortality and development of clinical signs of the disease. Any birds showing weight loss, leg paralysis and blindness were considered clinically positive for MD. However, the final conclusion was done only after necropsy by lesion detection specific for MDV infection. Each dead bird prior to the date of the final necropsy were subjected to a post-mortem examination to record the macroscopic lesions and cause of death.

RESULTS AND DISCUSSION

The results are presented in Table 1 and 2 and Figure 1.

The JM and RB1/B strains caused tumor development in 82% and 80% of the challenged chickens respectively (Table 1). Although there was no significant difference in tumor development between the two strains, RB1/B challenge strain caused significantly higher mortality in the challenged chickens (68% vs 61% with the JM strain). It was also observed that the tumor development in the JM challenged chickens were comparatively slower than the RB1/B challenged chickens, thus extending the date of final necropsy from 6 weeks post-challenge to 8 weeks. This could be because the JM strain is classified as a virulent pathotype (vMDV) whereas the RB1/B strain is classified as a very virulent pathotype (vvMDV).

It was interesting to observe that there was a distinct difference in the development and distribution of tumors in the chickens challenged with these two strains (Table 2). The RB1/B strain caused a significantly higher percentage of tumor development in the kidney, spleen, gonads, nerves, and skin. Whereas with the JM strain, a higher number of tumors were seen in the liver and heart. Tumors were seen in multiple organs of the chickens challenged with the RB1/B strain. Whereas, in the JM challenged chickens, tumors were mostly found in one or two organs.

The pathogenicity and infection rate of the JM and RB1/B strains have been compared previously. It is known that RB1/B is a very virulent strain that is more oncogenic and causes higher immunosuppression than the JM strain. However, in this study, we did not find any significant difference in the overall percentage of birds positive for MDV tumors between these two strains. Although JM had a slightly higher positivity, the lesions were less widespread throughout the bird with the majority of lesions confined to one organ. The birds infected with RB1/B had a higher percentage of multiple organ involvement (Fig. 1) and induced higher mortality than the JM strain. This aggressive nature of tumor development with the RB1/B strain is in agreement with most of the previous findings reported by previous authors, thus further proofing that RB1/B is more oncogenic than JM strain.

ACKNOWLEDGEMENTS

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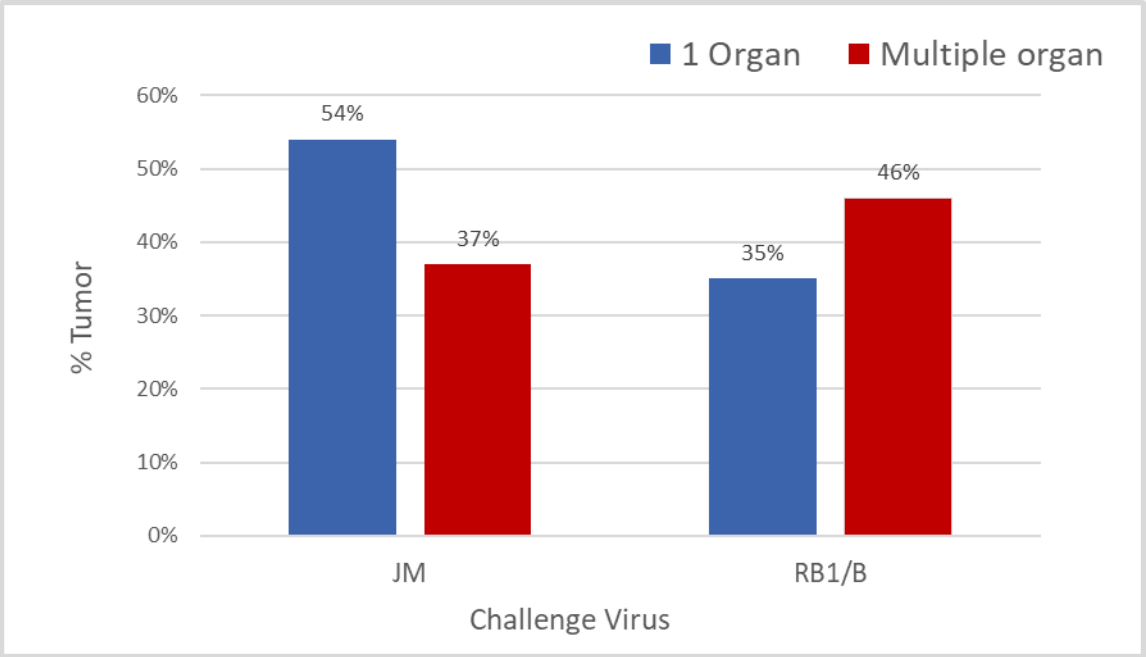
Table 1. Mortality and tumor development in the MDV challenged chickens.

Challenge virus	No. of trials	Total no. of birds	Total number positive for MD tumors	% Positive	Total mortality before final necropsy (%)
JM	10	220	180	82	61
RB1/B	8	160	128	80	68

Table 2. Lesion distribution in the MDV challenged chickens

Challenge virus	Tumor development (%)						
	Liver	Kidney	Spleen	Heart	Gonads	Nerve	Skin
JM	27	48	4	33	29	19	1
RB1/B	10	57	25	15	37	27	8

Figure.1. Number of organs showing tumors in the MDV challenged chickens.



GLOBAL UPDATES ON THE CURRENT HIGH PATHOGENICITY AVIAN INFLUENZA OUTBREAK AND ITS PHENOTYPES

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SUMMARY

Our knowledge of the ecology and epidemiology of avian influenza has changed over the past 150 years. There have been 44 genetically distinct high pathogenicity avian influenza (HPAI) epizootics since 1959 with the largest epizootic being caused by A/goose/Guangdong/1/1996 (Gs/GD) genetic Eurasian lineage of HPAI virus which began in 1996 and has evolved to multiple distinct genetic clades of virus. Over the past 2 decades, Gs/GD lineage of HPAI virus has evolved phenotypically in wild birds from resistance to infection to some viruses causing severe disease and mortality. This has been associated with five transcontinental movements of Gs/GD virus: 1) 2005 – clade 2.2; 2) 2007-2008 – clade 2.3.2.1, 3) 2014-2015 – clade 2.3.4.4c (previously terms 2.3.4.4A), 4) 2016-2017 – 2.3.4.4b, and 5) 2020-2022 – 2.3.4.4b. With the 2014 outbreak, intercontinental spread and extensive outbreaks of Gs/GD lineage HPAI occurred in poultry in North America, Africa, Middle Eastern and European countries. Historically, Gs/GD HPAI cases were initially H5N1, but assortment of the virus has produced H5N2, H5N3, H5N5, H5N6 and H5N8 HPAI viruses. Since 2014, the 2.3.4.4 clade viruses have diverged into eight genetic subgroups: a-h with major outbreaks of 2.3.4.4b occurring across Asia, Europe and Middle East in late 2020 and early 2021. Since 2020, most of the 2.3.4.4b viruses have been H5N1, H5N8 and H5N6 with H5N1 spread intercontinentally between Europe, and Africa, Asia and North America in late 2021 and early 2022, and late 2022 into Mexico and South American countries. To date, 2.3.3.4b virus has been detected in wild birds or poultry in Colombia, Ecuador, Peru, Chile and Venezuela. The current outbreaks include migratory aquatic birds, raptors, backyard and village domestic birds and commercial poultry.

First cases of H5N1 2.3.4.4b HPAI in North America occurred in Newfoundland, Canada, detected in samples collected from Great Black-Backed gulls and mixed poultry species from an exhibition flock in November and December 2021, respectively. With this Initial detection in Atlantic Flyway migrant aquatic birds, the virus spread southward in the winter 2022 in the USA, westward and northward in spring 2022, and return southward in fall 2022. The virus was rapidly detected in wide geographic areas in migratory aquatic birds, scavenger/predatory birds & resident aquatic bird populations. Over 50 different species of wild birds have been affected. By September 2022, the range of affected wild birds extended from Avalon Peninsula of Newfoundland in eastern Canada to the Aleutian Islands of the USA in the west to southern Florida in the USA in the south to Northern Slope of Alaska in the north. Beginning in October 2022, the virus was detected in Mexico and South America in wild birds and poultry.

There has been a geospatial association og outbreaks in commercial and non-commercial flocks of chickens, turkeys, domestic ducks, etc. with wild bird detections. As of 12-21-2022 in the USA, detections of the H5N1 HPAI virus include 5001 wild birds in 48 states, and 704 domestic bird premises (403 backyard flocks and 301 commercial flocks) in 47 states with 57.53 million domestic birds affected. In Canada, the virus has been detected in 1550 wild birds in 10 Provinces and 3 territories, and 274 domestic bird premises of 6.1 million birds in 9 provinces.

Since February, the HPAI outbreak has resulted in $2.26 billion is losses; $421 million in exports and $1.839 billion in drop in USA domestic value.

DUST AND ENVIRONMENTAL SAMPLES TO DETECT *ENTEROCOCCUS CECORUM* AND IDENTIFY POSSIBLE ENTRY SOURCES IN BROILER HOUSES

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SUMMARY

Environmental samples were collected on two different broiler farms and analyzed for the occurrence of *Enterococcus cecorum*. One farm had a known *Enterococcus cecorum* history, the other farm served as control farm without any clinical signs of *Enterococcus*-related diseases. Sets of samples were taken over the course of four production cycles (each of appr. 42 days). On day 1, 5, 10, 15, 21, 27, 34, 41, and in the service period, dust and fecal samples were taken from different locations following a defined sampling scheme. All samples were analyzed for *Enterococcus cecorum* by quantitative real-time PCR. Enterococcus cecorum was detected in samples from both farms, demonstrating the role of certain locations to serve as a reservoir for *Enterococcus cecorum*, even on farms where the birds show no clinical signs of disease.

INTRODUCTION

Enterococcus cecorum used to be considered as a non-pathogenic commensal and part of the regular gut microbiota in chicken. However, this has changed in recent years, and the species has become one of the most important pathogens in broiler chicken. Clinical signs of diseases are associated with pericarditis, perihepatitis, spondylitis, omphalitis, splenomegaly, ascites, osteomyelitis, and decreased performance, fatigue and progressive lameness can occur as consequences(1). Thus, *Enterococcus cecorum* leads to increasing mortality rates, increased production costs, and significant economic losses (2). Once *Enterococcus cecorum* infects a flock, there is a high possibility that subsequent flocks will also become infected via up to now mainly unknown reservoirs (2).

Since neither vaccination nor alternative treatment options are available, infections with Enterococcus cecorum are often treated with antibiotics. Due to emerging antibiotic resistances, the prudent use of antimicrobials is mandatory. For instance, Sharma et al. (3) showed that resistance genes against many common veterinary antibiotics are present in *Enterococcus cecorum* from both clinically healthy animals and diseased animals. Therefore, prevention of disease outbreaks by good hygiene practices and the interruption of infection chains are essential, also in the case of *Enterococcus cecorum*.

To achieve this goal, environmental samples were collected in this study to determine the localization and entry routes of *Enterococcus cecorum* on farm. The results can provide the basis for future projects to reduce the pathogen load, to prevent the risk of transmission to following flocks in the production cycle, and to find alternative preventive measures.

MATERIALS AND METHODS

Preparation. Prior to each sampling, 20 wipes (AlphaWipe® TX1004 Dry Cleanroom Wipers, Non-Sterile, Texwipe, Kernersville, United States of America) were moistened with 40mL PBS buffer, and 14 swabs (Alpha® Sampling Swab TX 715, Texwipe, Kernersville, United States of America) were moistened with 10 mL PBS buffer. Swabs and wipes were separately stored in different waterproof, autoclavable containers. The material was autoclaved and stored at four degrees before usage on farm.

Sampling. Two farms, one with a known history of *Enterococcus cecorum* infections, and the other with no clinical signs of *Enterococcus cecorum*-related diseases in the herds, were sampled over the course of four production cycles. On days 1, 5, 10, 15, 21, 27, 34, and 41 sampling took place via a standardized sampling scheme. For each defined location (e.g., ventilation system, drinking and feeding lines, boot profiles) either two wipes or two swabs

were used and pooled. Wipes were placed in Stomacher bags (Whirl-Pak® Standard Bags – 24 oz. (710mL), sterilized, Madison, United States of America) while swabs were stored in centrifuge tubes (Tube 50mL, 114x28mm, PP Sarstedt AG & Co. KG, Nümbrecht, Germany). On each day, two pooled fecal samples were taken. Additional samples were collected during each service period, e.g., inside drinking and feeding lines, on the floor, and behind power cable seals. Sock swabs (Sterisox tryptone complet, SodiBox, Pont C'hoat, France) were used in the service period to take samples from the floor before litter was spread and stored in a Stomacher bag. A total of 688 dust and fecal samples were collected.

Washing. After sample collection, the material was transported to the laboratory within three hours, and further stored at four degrees until further procession.

To extract the collected material from the wipes, 20 mL of PBS buffer was added to each Stomacher bag. The bags containing sock samples were washed out with 100 mL of PBS buffer. Each Stomacher bag was homogenized for two minutes at a speed of 240 RPM. The liquid from each bag was transferred into a 50 mL centrifuge tube. To the pooled swabs, 5 mL of PBS buffer was added and they were vortexed for one minute at 4,500 RPM. From each sample, 1 mL of liquid was transferred into a 1.5mL tube and centrifuged at 20,000 RPM. The supernatant was discarded and the pellet was transferred to DNA isolation.

The pooled fecal samples were mixed with a cotton swab each (Wattestäbchen 15cm steril, Metall, 2mm Kopf, WDT, Garbsen, Germany) for one minute. Afterwards, each swab was transferred into a 1.5mL tube. DNA isolation and PCR DNA was isolated from the swab samples, the wipes, the cotton swabs from the fecal samples and the sock samples. In this study, a commercial isolation kit (InnuPrep DNA Mini Kit 2.0, Analytik Jena AG, Jena, Germany) was used in accordance with the manufacturer's instructions.

Quantitative real-time PCR was performed for each sample in duplicate on 96-well-plates (Applied Bio-systems™, Fisher Scientific GmbH, Schwerte, Germany) using the QuantStudio 3 Real-Time-PCR-System (Thermo Fisher Scientific Inc.).

For this study, a cycle threshold (CT) value of 36 was used as cut-off, so that all results below this value were considered as positive. This method is highly specific, and documented to show reliable results for *Enterococcus cecorum* detection (4).

Results were statistically analysed with SAS Enterprise Guide (SAS Institute Inc., Cary, United States of America).

RESULTS

In this current study, over the course of four consecutive production cycles, 688 environmental samples were collected, and analyzed by real-time qPCR for *Enterococcus cecorum*. The final results of the still ongoing laboratory analyses will be presented at the congress. First results showed that *Enterococcus cecorum* was detected in the heat exchangers, in carcass garbage buckets and in dust on the feeding lines. Thus, risky locations can be defined which should be specifically considered when cleaning and disinfecting the barns. Furthermore, in a comparison of the samples of the two farms on the first day of production, in samples of the presumably *Enterococcus cecorum*-free farm *Enterococcus cecorum* was detected. On the farm with the documented *Enterococcus cecorum* history, on the other hand, all environmental samples were negative on the first day of the first production cycle.

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IMPACT OF *EIMERIA MELEAGRIMITIS* ON THE ILEAL AND CECAL MICROBIOME IN COMMERCIAL TURKEY HENS

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ADENOVIRAL PROGENY PROTECTION RESULTS

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ABSTRACT

Inclusion body hepatitis (IBH) have increased in broilers over the past 5 years. Aviadenoviruses isolated/ detected from IBH cases primarily belong to FAV group E, FAdV 8b. Many companies utilize adenovirus isolates from clinical case submissions in autogenous vaccines in pullets to prevent vertical transmission and provide maternal antibodies to progeny. FAV virulence varies by and within serotypes. This study evaluated the pathogenicity of FAVE, FAdV 8b IBH field isolate in specific pathogen free (SPF) chicks. Mortality and gross and microscopic liver lesions observed in inoculated chicks. In a follow-up vaccination protection study, progeny from adenovirus FAVE/8a, FAVE/8b and FAVD/11 autogenous vaccinated breeders were challenged with this contemporary FAVE, FAdV 8b field isolates, or FAV D, FAdV 11 or FAV E, FAdV 8b challenge viruses to determine whether vaccination antibodies provided protection. No clinical disease, gross or microscopic lesions observed in any challenged flocks suggesting the autogenous vaccine provided protection.

AVIARY SYSTEM MANAGEMENT ISSUES LEADING TO AN UNCOMMON LESION

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SUMMARY

Eight live organic layers, 18 weeks of age, displaying signs of morbidity were necropsied on the farm. Formalin fixed samples were submitted to the Poultry Diagnostic and Research Center with a history of high mortality since the move to the lay house. The flock also had a history of poor performance in the pullet house. Seven out of eight had severe muscle atrophy, lack of body adipose tissue and no ovarian development. There was no feed in the digestive tract or litter. Histopathology results include catarrhal tracheitis, multifocal granulomatous myositis, mild multifocal lymphocytic interstitial nephritis and severe diffuse serous atrophy of fat in the bone marrow. The flock failed to access all levels of the pullet and subsequently the lay aviary system resulting in severe emaciation.

INTRODUCTION

Gelatinous bone marrow transformation (GBMT) otherwise known as serous atrophy of fat or starvation marrow is characterized by atrophy of adipose tissues, loss of hematopoietic cells and extracellular deposition of mucopolysaccharides rich in hyaluronic acid (1-3). This condition has been well documented in people (4-12) with numerous underlying conditions and in various mammal species like cats (13), mini pigs (14), cattle (15), reindeer (16), a mini horse (17) and rabbits (18). Most of these cases were associated with gastrointestinal disease that prevented absorption of nutrients or inadequate nutrition or inanition. There is a report of 33 Whooper Swans with lead poisoning in Japan (19) where 7/14 swans were noted to have gelatinous bone marrow grossly and 13/13 with mild to moderate hypoplasia of the bone marrow microscopically. The pathogenesis of lead toxicity on erythropoiesis has been well documented (19). This case report examines an organic layer flock with a history of poor performance after moving to the lay aviary system.

METHODS AND MATERIALS

Eight live 18-week-old organic layers displaying signs of morbidity were necropsied on the farm. Gross lesions included 7/8 with severe muscle atrophy, loss of body adipose tissue and no ovarian development. There was no feed or litter in the digestive tracts. One bird had been caught in the housing system and euthanized. This bird had trauma noted on the right thigh with hemorrhage and edema. Formalin fixed tissue samples of bones, skeletal breast muscles, tracheas, livers, spleen, hearts, kidneys, and duodenums with pancreas were submitted to the Poultry Diagnostic and Research Center Diagnostic Laboratory for microscopic analysis.

Routine tissue processing, embedding, sectioning and hematoxylin and eosin staining was performed on submitted tissue and then examined by light microscopy. Bones underwent decalcification before processing in order to section. Perl's iron histochemical staining was performed on the liver and spleen sections.

RESULTS

The most significant lesions were in the bones and skeletal muscles. The bones had adequate cortical bone thickness in all 7 sections. Bone marrow spaces have abundant eosinophilic fluid replacing adipocytes. There are little to no lymphocytes present in the spaces. Granulocytic cells and precursors are present along with mature RBCs in severely decreased amounts (Figures 1a and 1b). One section of skeletal muscle had a few granulomas characterized by central cellular debris admixed with large clear spaces that are surrounded by multinucleated giant cells and epitheloid macrophages and lymphocytes located along the edge of the tissue. One section has several similar granulomas on the edge and into the muscle bundles. Two section have scattered fibers that are hypereosinophilic and rarely fragmented.

Additional microscopic lesions were found in the remaining organs. The kidneys had one section with multiple areas of mild lymphocytic infiltrates in the interstitium and some lymphocytes in a medullary cone. One other section

has multiple areas of mild lymphocytic infiltrates in the interstitium. The liver sections had one with multiple areas of fibroplasia around portal triads and several fibrin thrombi in the parenchyma. Three sections have scattered Kupffer cells with amber pigments that were positive for hemosiderin by Perl's iron stain. One section had multiple areas of macrophages in aggregates throughout the parenchyma. The tracheas had 2 sections with loss of cilia in multiple areas and excess mucin in the lumen. All pancreatic sections had scattered pyknotic cells in the exocrine gland tissue and zymogen granules were diffusely decreased in numbers. The spleen had a mild decrease of lymphocytes in the periarteriolar sheaths and there were scattered macrophages with amber pigments that stained positive for hemosiderin by Perl's iron. The hearts had one section a focal area of mild lymphocytic infiltrates and a focal area of mild heterophilic infiltrates on the epicardium. Another section had a focally extensive area of fibrin and edema with scattered heterophils on the epicardium.

DISCUSSION

The microscopic lesions in the bones are consistent with gelatinous bone marrow transformation or more commonly known as serous atrophy of fat. This in combination with the gross lesions of severe skeletal muscle atrophy and lack of body adipose tissue is consistent with severe emaciation. The pancreatic changes with loss of zymogen granules also supports emaciation. Discussions with the grower included helping the birds reach the feeders by placing more ramps and walking the house to encourage birds to go up the ramps in order to get access to feed and water or place them into the system for that access. Additional history revealed that water consumption for the flock was less than expected and birds did not access all levels of the pullet aviary system. When moving to the lay facility, the flock continued to struggle to access levels of the aviary system. Birds failing to access levels of the aviary system thus failed to access feed and/or water. Supplemental waterers were placed on the floor in response, however that lack of feed access for an extended period of time led to the significant microscopic lesions in the bone marrow and the zymogen granule atrophy in the pancreas.

The additional microscopic lesions seen can be due to a variety of reasons. The granulomatous myositis was consistent with killed vaccine administration while the remaining degenerative myopathy has several etiologies with Vitamin E/selenium deficiency being most likely. Kidney lesions were consistent with IBV vaccination/or exposure with one section having lesions highly suggestive of nephropathogenic IBV. The presence of hemosiderin laden macrophages in the livers and spleen indicate RBC death which was increased in this case due to the emaciation and suspect bacterial infection/toxins (other liver changes). The loss of tracheal cilia and excess mucin is highly suggestive of high levels of ammonia in the environment.

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Figure 1a. Bone marrow. Diffusely there is loss of cellularity of marrow components, atrophy of adipocytes and abundant amounts of eosinophilic material present (edema fluid). H&E.

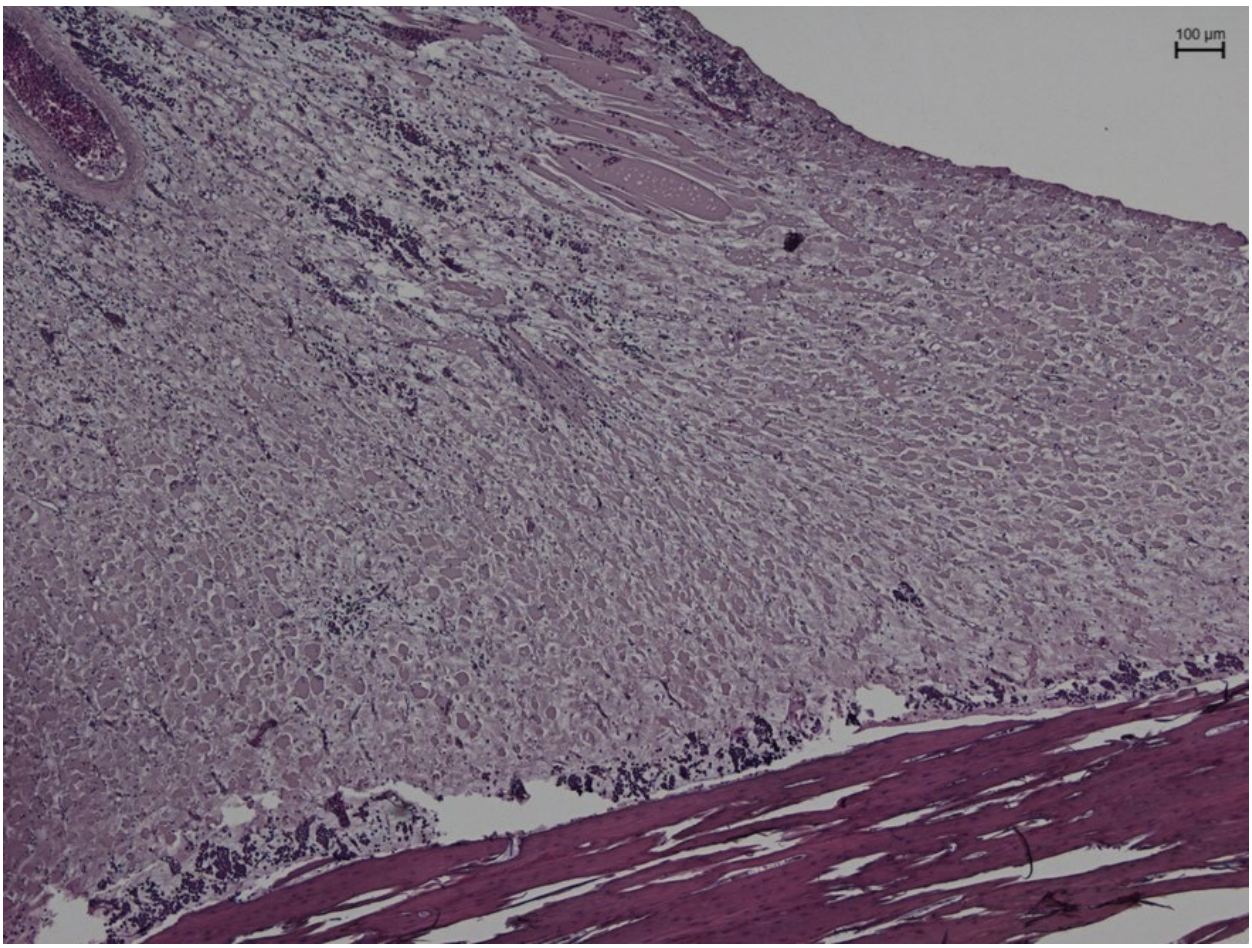

Figure 1b. Bone Marrow. Note the amount of eosinophilic material and atrophy of adipocytes (large clear circles) along with the lack of hematopoietic stem cells.