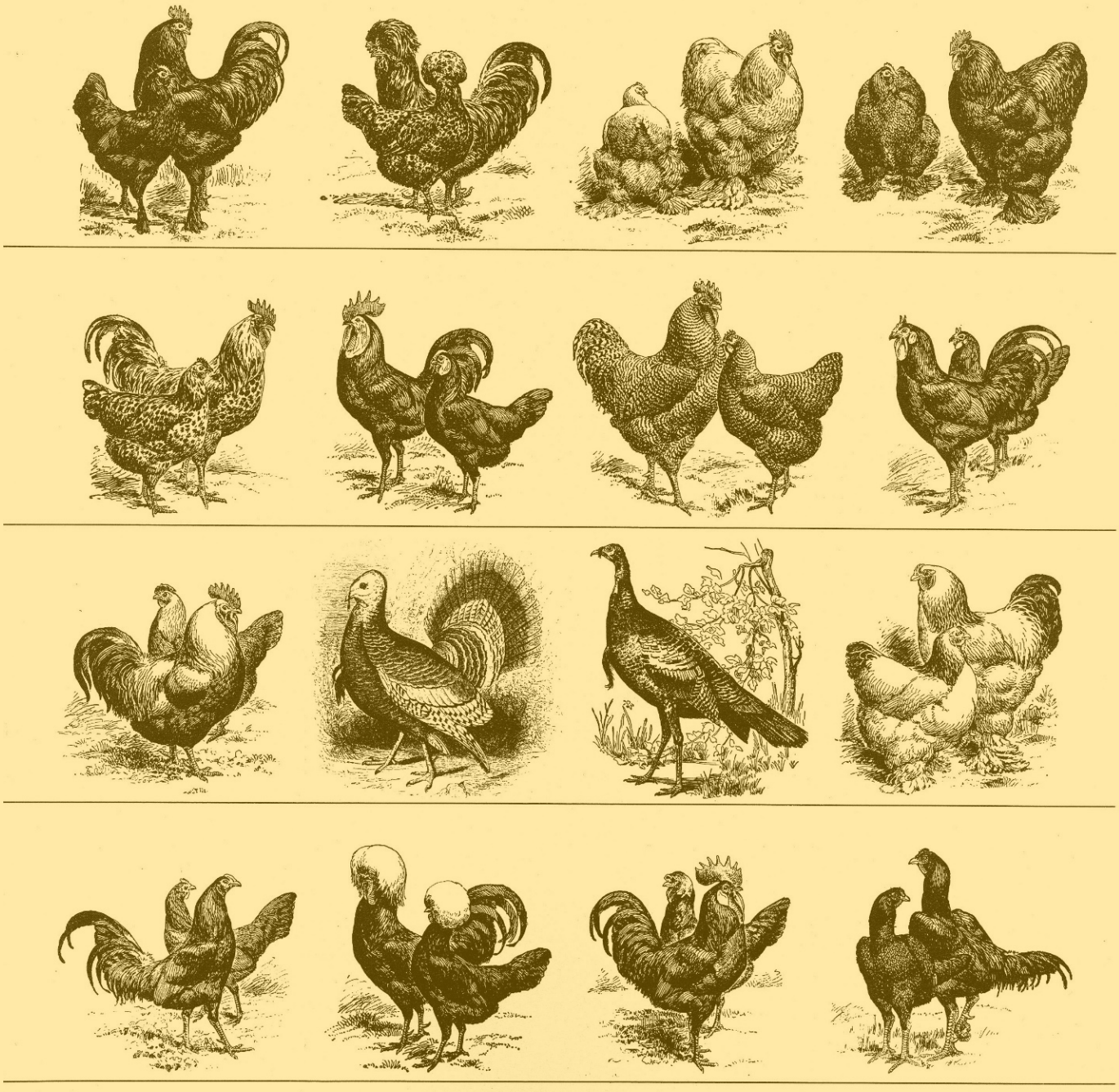
PROCEEDINGS OF THE SIXTY-SEVENTH WESTERN POULTRY DISEASE CONFERENCE

April 16-18, 2018 Salt Lake City, UT



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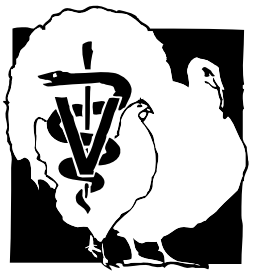

Western Poultry Disease Conference
Special Recognition Award

David D. Frame, DVM, DACPV

The 67th WPDC is honored to present the WPDC Special Recognition Award to Dr. David D. Frame.

Dr. David Frame was born (in July 1955) and raised in Kearns, Utah. As a youth, Dave developed an interest in birds. Along with his dad, he designed an extensive breeding and growing program for exhibition chickens. Dave continues to be involved with judging of these birds at various county and state fairs.

David graduated from Utah State University (USU) in 1980 with a B.S. in Animal Science. He was then accepted into the second class of veterinary students at Oregon State University (OSU). At that time, vet students had to spend over a year at Washington State University in Pullman, WA, to receive the necessary training in small animal medicine and surgery. In 1984, Dave received his Doctor of Veterinary Medicine (DVM) degree from Oregon State University, and subsequently, completed the 2-year residency program in avian medicine at the University of California, Davis. Dave is grateful to be able to spend time with Dr. Art Bickford while based in the Turlock laboratory, as one of his current roles is Avian Veterinarian at USU's Central Utah Veterinary Diagnostic Laboratory.

Upon completion of his residency, Dave moved back to Utah and began his career as the Chief Veterinarian for Moroni Feed Company. For 12 years, he was responsible for all turkey health programs and disease diagnostics, for both breeder and meat turkeys, at Moroni Feed Company.

In 1998, David accepted the position of Extension Poultry Specialist for USU. In this role, he supports and educates small flock and backyard poultry owners, game bird producers, and commercial turkey and chicken growers in poultry health and management. Additionally, he is an associate professor in the USU department of Animal, Dairy, and Veterinary Sciences, and actively participates in providing community education classes in backyard

poultry raising. And, for his third and final role, Dr. Frame serves as the Avian Veterinarian in USU's Central Utah Veterinary Diagnostic Laboratory.

David's contribution and service to the industry has not gone unrecognized. He was awarded a Service Appreciation award for 3 consecutive years (2004-2006), and was recently honored as "Scientist Of The Year" in 2016, by the Pacific Egg & Poultry Association.

David has participated in the Western Poultry Disease Conference since 1985. Of course, his most memorable and "earth-shaking" WPDC meeting was in 1986 in Puerto Vallarta!?! In 2000, Dave accepted the position of WPDC's Proceedings Editor, and has since provided 18 years of outstanding service. He continued to improve the quality of the proceedings and to maintain it as one of the most referenced publication of meetings specializing in poultry medicine. He has led the digitization of the proceedings from print to CDs, to flash drives, and finally, to web-based publication.

David and Lisa met at USU and were married in 1979. They have four children and love spending time with their four grandchildren. David spent his mission in Honduras and Nicaragua, where he became fluent in Spanish and learned to love hot and spicy food! David enjoys hiking in southern Utah, geology, bird watching and identifying bird calls. He enjoys gardening and has been breeding his own line of Indian corn for many decades. Every Halloween, neighbors come to see David's front porch, which he turns into an elaborate seasonal display that he redesigns every year. And of course, there are the SF Giants.

The 67th WPDC is honored to recognize Dr. David D. Frame for his service and advancement of knowledge of avian diseases in the western region, and for his 18 years, and counting, as Proceedings Editor for the WPDC.

67th WPDC CONTRIBUTORS LIST

(As of April 5, 2018)

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Moroni, UT

David D. Frame, DVM
Ephraim, UT

SPECIAL ACKNOWLEDGEMENTS

The 67th Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and to help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. More than 30 organizations, companies and individuals have once again given substantial financial support. Many companies and organizations, including some that also contribute financially, send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

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

Dr. Rodrigo Gallardo, Program Chair of the 67th WPDC, would like to thank the School of Veterinary Medicine at UC Davis, for the support and trust given as the head of the Poultry Medicine Program. His team at the Poultry Medicine Laboratory Ana Paula da Silva, Alejandra Figueroa, and Sofia Egana for administrative and technical help. Most importantly to his family his wife Gigi and two kids Matias and Emma for supporting him at all times.

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

For the past 21 years, Dr. Chin served as secretary-treasurer of the WPDC. He has decided it is time to hand it over to the younger folks, so, this was his last year as secretary-treasurer. There are so many people he wants to thank for their support, including all the contributors, sponsors, presenters, administrative staff and attendees. These folks are the ones who are truly responsible for the continual growth and success of the WPDC. Rich would like to acknowledge and thank Art Bickford, Dick Yamamoto, and Rosy for their unwavering support, guidance, and "convincing" him to serve as secretary-treasurer. Rich is extremely grateful to Dave Frame who was equally responsible for the success of WPDC. Finally, Rich would like to thank his wife, Elaine, for allowing him to spend the numerous hours working on the WPDC, especially talking with Rosy on the phone! Yes dear, now it's time for that "honey-do" list…

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.

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Dr. Richard P. Chin
rpchin@ucdavis.edu

67th WPDC PROCEEDINGS

Please note that the proceedings of the 67th 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 67th WPDC are available in electronic format only. They can be downloaded from the American College of Poultry Veterinarians website (www.acpv.info).

WESTERN POULTRY DISEASE CONFERENCE (WPDC) HISTORY

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

YEAR	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
35th WPDC – 1986	D. W. Waldrip	Duncan A. McMartin	J. A. Allen	
11th ANECA	Jorge Basurto	Mario Padron	A. Tellez-G. Rode	
36th WPDC – 1987	D. A. McMartin	Marcus M. Jensen		
37th WPDC – 1988	M. M. Jensen	Barry Kelly	A. S. Rosenwald	
38th WPDC – 1989	B. Kelly	Masakazu Matsumoto		Louise Williams
39th WPDC – 1990	M. Matsumoto	Jeanne M. Smith		Dean Young
40th WPDC – 1991	J. M. Smith	Richard P. Chin	A. S. Rosenwald	
16th ANECA	Martha Silva M.	David Sarfati M.	A. S. Rosenwald	
41st WPDC – 1992	R. P. Chin	Rocky J. Terry	Marcus Jensen	Henry E. Adler *
				*(posthumous)
				R. A. Bankowski
				C. E. Whiteman
42nd WPDC – 1993	R. J. Terry	A. S. Dhillon	W. W. Sadler	Royal A. Bagley
43rd WPDC – 1994	A. S. Dhillon	Hugo A. Medina		G. B. E. West
44th WPDC – 1995	H. A. Medina	David D. Frame	W. M. Dungan*	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 Chin
67th WPDC – 2018	C.G. Sentíes-Cué	Rodrigo A. Gallardo		

MINUTES OF THE 66TH WPDC ANNUAL BUSINESS MEETING

President Susantha Gomis, called the meeting to order on Monday, March 20, 2017, at 4:20 PM, at the Holiday Inn Downtown Arena hotel. There were 16 people in attendance.

APPROVAL OF 65TH WPDC BUSINESS MEETING MINUTES

The minutes of the 65th WPDC business meeting was reviewed by members of the Executive Committee minutes during the Executive Committee meeting and recommended approval as written.

ANNOUNCEMENTS

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

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

It was noted that Duncan McMartin passed away since our last conference.

REPORT OF THE SECRETARY-TREASURER

Dr. Chin presented the Secretary-Treasurer report. For the 2016 meeting, we had an income of approximately $80,000 and a expenses of approximately $92,000, which resulted in a net loss of approximately $12,000. The loss was due to increases in travel and hotel (food, guest rooms and audiovisual) expenses, and the purchase of two laptop computers ($700 each) for use by those in the Executive Committee and for presentations at the meeting.

Dr. Chin noted that expenses continue to increase and suggested that it was time to increase registration fees, except student fees, by $25. During the discussion, it was suggested that we also increase the late fee for presenter registration. One person asked if we should charge the regular registration fee to everyone, including presenters. A motion was made to increase registration fees (presenter, non-presenter, early and late) by $25. Rates were previously increased in 2011. [Note: Following additional discussion, the WPDC Executive Committee decided to increase fees for 2018 to $260 for early regular registration, $315 for late registration, $180 for presenters and $100 for students. They also recommend increasing fees again in 2020.]

REPORT OF THE PROCEEDINGS EDITOR

Dr. David Frame presented the Proceedings Editor report. There were 76 papers submitted for publication in the proceedings, and the process went relatively smoothly. He thanked the authors for their timely submissions. It was suggested that we also provide an easier process for submitting papers.

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

FUTURE MEETINGS

President Gomis reported that ANECA proposed a joint meeting for 2019 in Puerto Vallarta. Dr. Chin noted that the WPDC current rotation is for the 2019 meeting in Sacramento and the 2020 meeting in Mexico. Following a

brief discussion, a motion was made and approved to have a joint meeting with ANECA in Puerto Vallarta, Mexico, in 2019. The following schedule was tentatively set:

2018: 67th WPDC, Salt Lake City, April 15-18, 2018.

2019: 68th WPDC (joint meeting with ANECA), Puerto Vallarta, Mexico, April 13-17, 2019

2020: 69th WPDC, Sacramento, CA (to be determined)

People were reminded that they vote on the locations each year, so it can be changed. Dr. Chin reminded the group (in particular, the next secretary-treasurer) that potential hotels need to be contacted 2-3 years in advance. Dr. Chin will contact the Holiday Inn in Sacramento to cancel the reservations made for 2019 and look into 2020. [Note: The Holiday Inn asked to be contacted again in 2018 for a meeting in 2010.]

WPDC EXECUTIVE COMMITTEE

Dr. Sarah Mize volunteered to be Program-chair for the meeting in 2019 (the joint meeting with ANECA).

There were no other nominations and Dr. Mize was elected unanimously as program chair-elect. The following officers were nominated for 2017-2018:

Program Chair: Dr. Rodrigo Gallardo

President: Dr. Gabriel Senties-Cué

Past-President: Dr. Susantha Gomis

Contributions Chair: Dr. Yan Ghazikhanian

Proceedings Editor: Dr. David Frame

Secretary-Treasurer: Dr. Richard Chin

Program Chair-elect: Dr. Sarah Mize

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

NEW BUSINESS

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

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

Dr. Senties-Cué adjourned the 66th WPDC annual business meeting at 4:47 PM.

TABLE OF CONTENTS

LIPOSOMAL ASPERGILLUS PROTEIN VACCINE EFFECTIVE IN PREVENTING PULMONARY ASPERGILLOSIS IN SPF CHICKENS

M. Slarve, H. Reza, and J. Adler-Moore

Biological Sciences Department, Cal Poly Pomona, Pomona, CA. 91768

BACKGROUND

Aspergillus molds, most commonly *Aspergillus fumigatus*, are ubiquitous in the environment due to their airborne spores, which when inhaled are capable of causing severe infection in a wide range of species, primarily targeting the lungs. In humans, this pulmonary infection generally only affects immunosuppressed patients. Chickens and turkeys are also susceptible when they are stressed. *Aspergillus* spp are commonly found in high quantities in turkey and chicken houses in the air, bedding, litter, and feed (1). The dust from litters and feeds contains the spores, which are scattered in the environment (2). During inhalation by poultry, *A. fumigatus* conidia (2-3um), are able to travel throughout the respiratory system due to the low concentration of ciliated columnar cells in their lungs (1). The spores penetrate into the aerial apparatus, invading the lungs, tracheas and air sacs, but in the systemic form of the disease, the fungus can also affect the liver, the kidney, the encephalon, the bones, the skin and the eyes (2).

Pulmonary aspergillosis causes acute symptoms in young birds with a high mortality rate of up to 90%, leading to carcass condemnation, and loss of entire flocks of livestock (5). Logistical complications in delivering an antifungal drug to a flock of chickens, as well as increasing restrictions on the administration of antimicrobial drugs to livestock makes treating the infected birds challenging and inefficient (6). A vaccine conveying protection against *A.fumigatus* spores could effectively prevent the establishment of the infection, and avoid the heavy economic losses that it causes. No such vaccine is currently commercially available.

To address this problem, we have been using a liposomal Aspergillus vaccine prepared by Molecular Express Inc. (VesiVax®) to test its efficacy against pulmonary aspergillosis in SPF chickens. The vaccine contains the Aspergillus protein Aspf3, which is a two-cysteine peroxiredoxin enzyme that is abundant in the fungal conidia both intracellularly and extracellularly. It functions to protect the conidia from oxidation by reactive oxygen species. When this protein is genetically knocked out, the resultant strain is rendered non-pathogenic (4). It also contains the Aspergillus protein Aspf9 which is a cell wall glucanase occurring on the surface of the conidia (3).

METHODS AND RESULTS

To initiate our studies, we had to first standardize a pulmonary aspergillosis infection in SPF chickens. Variables investigated included fungal spore challenge doses (5 X 10ex7, 1 X 10ex8, 4 X 10ex8) and the immunosuppressive regimen (3 or 4.5 mg/kg dexamethosone) since immunosuppression is a major risk factor for aspergillosis even in birds. SPF chickens (male and female, 42 days old, n=7/group) were administered dexamethasone intramuscularly at 3 mg/kg or 4.5 mg/kg on day -1, day 0, day +1 and day +2 relative to nasal/intraocular *A. fumigatus* challenge (Strain ATCC 045) on day 0. We used a dose of 4 X 10ex8 viable Aspergillus spores/chicken in 200 uL, as a previous study showed that this spore dose would be more effective than lower spore doses in producing the infection. We validated the spore dose by plating a sample of the inoculum on Sabouraud's dextrose agar. Chickens were monitored for weight loss following *A. fumigatus* challenge and day +7 post-challenge, they were euthanized for collection of lungs and tracheas. The tissues were weighed and then homogenized with a probe homogenizer in 2 mL of PBS with 0.05% chloramphenicol. Homogenates were serially diluted in PBS with 0.05% chloramphenicol and 400 uL aliquots plated in duplicate onto Sabouraud's dextrose agar. The plates were incubated at 37°C for 24 hr and colony forming units (CFU) counted to obtain CFU/g of lung or trachea. The results of this study showed that when the chickens were immunosuppressed with 3 mg/kg or 4.5 mg/kg dexamethasone, they had a high fungal burden in the lungs and tracheas, with the 4.5 mg/kg dexamethasone dose producing a more severe infection in the chickens than the 3 mg/kg dexamethasone.

This pulmonary aspergillosis infection model was then used in a vaccine efficacy experiment to test how well the liposomal Aspergillus vaccine would protect against infection. The SPF chickens (male and female, n=7/group) were vaccinated with 200 uL of phosphate buffered saline (PBS, control group) or a mixture of VesiVax liposomes (Molecular Express

Inc.) containing 100 uL of liposomes with Aspf3 (7.5 ug/dose) and 100 uL of liposomes with Aspf9 (7.5 ug/dose), and the adjuvant monophosphoryl Lipid A (MPL, 15 ug/dose) or the adjuvant lipidated tucaresol (5 ug/dose). The priming vaccine dose was given to 10 day old chickens subcutaneously followed by nasal/intraocular boosts when the chickens were 21 days old and again when they were 42 days old. The chickens were then immunosuppressed intramuscularly with 4.5 mg/kg dexamethasone on day -1, day 0, day +1 and day +2 relative to nasal/intraocular *A. fumigatus* challenge (Strain ATCC 045) on day 0 with 4 X 10ex8 viable Aspergillus spores/chicken. The birds were 49 days old on the day of challenge. Following challenge, they were monitored for weight loss and day +7 post-challenge, they were euthanized for collection of lungs and tracheas that were processed as described above for determination of fungal burden (CFU/g tissue). The results of this study showed that chickens vaccinated with a mixture of Aspf3 and Aspf9 liposomes containing either MPL or lipidated tucaresol adjuvant had much better survival (100%) when compared to the PBS control group (63%). Birds vaccinated with the Aspergillus liposomes had reduced fungal burden in their lungs and significantly ($p \leq 0.048$) reduced fungal burden in their tracheas when compared to the PBS control (Figure 1).

To further examine the efficacy of the Aspergillus vaccine, we tested its protection against pulmonary aspergillosis in younger, broiler age chickens which were 28 days old on the day of fungal challenge. SPF chickens (male and female) were vaccinated subcutaneously at 7 days of age (priming dose) and then given intranasal/ocular boosts at 14 and 21 days of age. The chickens (n=8/group) were given either 200 uL of PBS, 200 uL liposomes with the adjuvant lipidated tucaresol (5 ug/dose) and no Aspergillus proteins, or a mixture containing 100 uL of liposomes with Aspf3 (7.5 ug/dose) and 100 uL of liposomes with Aspf9 (7.5 ug/dose) and the adjuvant lipidated tucaresol (5 ug/dose). At 27, 28, 29 and 30 days of age (Day -1, 0, +1, and +2 relative to challenge), the chickens received 4.5 mg/kg of dexamethasone via intramuscular injection. At 28 days of age, the birds were challenged with 2.25 X 10ex8 viable *A. fumigatus* spores/chicken (Strain ATCC 045) given by intranasal/intraocular administration in a volume of 300 uL. The chickens were monitored for weight change for five days post challenge and euthanized on day +5 post-challenge for collection of blood, lungs, and trachea. A portion of each tissue for six birds/group was processed for histopathology hematoxyliin and eosin staining. The remaining tissues were homogenized and plated for determination of fungal burden as described above. The serum from the blood was used to determine the anti-Aspergillus antibody agglutination titers for each group, with the *A. fumigatus* spores as the test antigen.

Results with the younger chickens paralleled what we had observed in the older chickens. The Aspergillus liposome treated group had significantly lower weight loss compared to the birds given liposomes with only lipidated Tucaresol (p=0.0003) or PBS (p=0.0104). With respect to fungal burden, significantly lower CFU/g in the lungs (Figure 2) and tracheas were detected in the Aspergillus liposome group versus the PBS control group (p=0.0070 lungs and p= 0.0002 tracheas, respectively); the Aspergillus liposome group also had lower CFU/g in the lungs than the birds given liposomes with only lipidated tucaresol (p=0.0030). There was significantly higher spore agglutination titers in the serum of fungal challenged chickens that received Aspergillus liposome treatment (mean = 426) versus treatment with PBS (mean = 40, p=0.0012) or liposomes with lipidated tucaresol only (mean =27, p=0.0006). The histopathology data showed that the Aspergillus vaccine induced limited granuloma formation in the lungs with the PBS group producing mild granuloma formation, and the group given liposomes with only lipidated tucaresol having large granulomas with multinucleated giant cells with fungal elements.

CONCLUSIONS

The potential for this vaccine to be an effective method of protecting poultry from pulmonary aspergillosis is supported by the significant protection we observed in chickens following vaccination with a VesiVax liposome formulation containing the immunogenic Aspergillus proteins, Aspf3 and Aspf9, and either MPL or lipidated tucaresol as adjuvants. By using this vaccine, the incidence of pulmonary aspergillosis could be markedly reduced, and that could result in a significant reduction in costs for the poultry industry.

REFERENCES

1. Arné, P., S. Thierry, D. Wang, M. Deville, G. Le Loc'h, A. Desoutte, and J. Guillot (2011). *Aspergillus fumigatus* in poultry. International Journal of Microbiology. [Internet]. Available from: http://doi.org/10.1155/2011/746356. 2011.

2. Cacciuttolo, E., G. Rossi, S. Nardon, R. Legrottaglie, and P. Mani. Anatomopathological aspects of avian aspergillosis. Veterinary Research Communications. 33(6): 521–527. 2009.

3. N. Chaudhary, J. F. Staab, and K. A .Marr. Healthy human T-cell responses to *Aspergillus*

fumigatus antigens. PLoS ONE. 5(2): e9036. doi:10.1371/journal.pone.0009036. 2010

4. Hillman F. Et al. The Crystal Structure of Peroxiredoxin Aspf 3 Provides Mechanistic Insight into Oxidative Stress Resistance and Virulence of *Aspergillus fumigatus*. Scientific Reports, Nature. Vol 6:33396 e Pub. 2016.

5. Latgé, J. P. *Aspergillus fumigatus* and aspergillosis. Clinical Microbiology Reviews, 12(2): 310–350. 1999.

6. SB-27 (Senate Bill No. 27). Livestock: use of antimicrobial drugs-California bill. 2015-2016.

Figure 1. CFU/g trachea following *Aspergillus fumigatus* challenge of SPF chickens (male and female) 49 days old, previously vaccinated with Phosphate buffered saline (PBS) or liposomes containing the *Aspergillus* proteins Aspf3 and Aspf9 and the adjuvant monophosphoryl lipid A (MPL) or lipidated tucaresol (LT1).

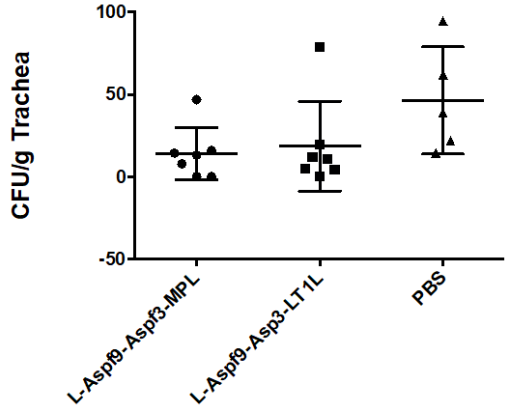
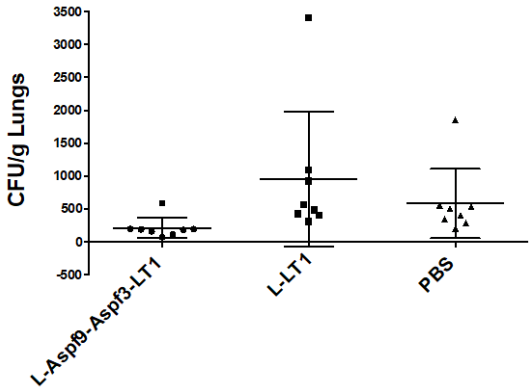


Figure 2. CFU/g lungs following *Aspergillus fumigatus* challenge of SPF chickens (male and female) 28 days old, previously vaccinated with Phosphate buffered saline (PBS), liposomes with lipidated tucaresol (LT1) and no Aspergillus proteins, or liposomes containing the Aspergillus proteins Aspf3 and Aspf9 and the adjuvant LT1.



ANTIMICROBIALS USED FOR THE THERAPY OF NECROTIC ENTERITIS AND COCCIDIOSIS IN BROILER CHICKENS AND TURKEYS IN CANADA, PREVENTIVE APPROACHES AND FLOCK HEALTH IMPLICATIONS

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SUMMARY

Since 2013, the farm component of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) has been collecting antimicrobial use (AMU) and antimicrobial resistance (AMR) data from sentinel broiler chicken flocks (five provinces) and sentinel turkey flocks (initially in one province from 2013-15; increasing to two provinces for 2016). Producers, through their veterinarians, voluntarily provided data and enabled collection of samples for bacterial culture and susceptibility testing. The objectives of CIPARS Farm Surveillance are to provide data on AMU and AMR, to monitor temporal and regional trends in the prevalence of AMR, to investigate associations between AMU and AMR, and to provide data for human health risk assessments. Between 2013 and 2016, a total of 509 broiler flocks and 160 turkey flocks were surveyed. Questionnaires were used to collect information on AMU and farm-level operational factors. Overall, antimicrobials used for the prevention of *Clostridium perfringens* infections (necrotic enteritis) and ionophores and chemical coccidiostats for the prevention of coccidiosis contributed to >90% of the quantity of antimicrobials (kg, mg/PCU) administered via feed for both species. Evaluation of farm-level operational factors may be useful in managing these enteric diseases and informing further AMU reduction initiatives and the use of other non-antimicrobial alternatives.

METHODS

General surveillance design. CIPARS monitors AMR and AMU through a framework of fifteen purposively selected sentinel poultry veterinary practices located in the major poultry producing provinces across the country (British Columbia, Alberta, Saskatchewan, Ontario, and Québec). These veterinary practices utilized specific inclusion/exclusion criteria to enroll broiler chicken and turkey producers as described elsewhere (1). Allocation of flocks per province was proportional to the number of producers or provincial flock populations. The exception to this approach was that in the three provinces where CIPARS with FoodNet Canada (British Columbia, Alberta, Ontario), a minimum of 30 flocks were sampled; regardless of broiler flock numbers or provincial flock populations.

Farm data collection and analysis. Data on AMU by route of administration, and biosecurity and flock health were collected by questionnaire at each sampling visit. Data were entered into a PostGreSQL Database and descriptive statistics were obtained with Microsoft Excel (Office 14), StataSE Version 15 (College Station, Texas) or SASv9.3 (Cary, North Carolina). Antimicrobial and coccidiostat exposures were summarized by active ingredient or antimicrobial class and also summarized quantitatively by class or as an aggregate of all the classes reportedly used.

The following operational/farm factors were examined with respect to coccidiosis status: antibiotic free (ABF) status or raised without antimicrobials, AMU, downtime, cleaned and disinfected the barn (C & D) prior to chick placement, barn population, stocking density, chick source (imported or domestic chicks), all in-all out, season, age of flock and coccidiosis vaccination. A negative binomial model was used to assess the factors listed above as well as the following variables: laboratory *Salmonella* status, diseases diagnosed and vaccination status (e.g., Infectious Bursal Disease). StataSE Version 15 was used for data analysis.

Quantitative estimates. Using methods adapted from the the European Surveillance for Veterinary Antimicrobial Consumption approach (2), the quantity of antimicrobials in mg active ingredient adjusted for population and weight, expressed as mg/PCU (Population Correction Unit), was determined for each antimicrobial class and total AMU. Average weight at treatment for calculating mg/PCU was 1 kg for broiler chickens and 6.5 kg for turkeys. The days at risk (average age of the flock at pre-harvest visit) used was 33 days for broilers, and 84-88 days for turkeys depending on the year.

Details regarding methods used for data collection and analysis are described elsewhere (1).

RESULTS AND DISCUSSION

Descriptive information. Between 2013 and 2016, a total of 509 broiler flocks and 160 turkey flocks were surveyed. A proportion of the flocks surveyed were under an antibiotic-free or raised without antibiotic program (broilers: 8%, turkeys: 7%). The total biomass during the study timeframe was 11.7 million kg and 8.8 million kg for broiler chickens and turkeys, respectively.

Antimicrobial use. Overall, antimicrobials used for the prevention of necrotic enteritis (NE) and coccidiosis accounted for 95% and 99% of the quantity of antimicrobials (kg, mg/PCU) administered via feed for broiler chickens and turkeys, respectively. The remaining 1–5% were used for the treatment of localized and systemic infections (e.g., avian pathogenic *E. coli*, staphylococcal infections, other clostridial infections such as dermatitis, and *Enterococcus cecorum*).

Broiler chickens. For coccidiosis prevention, 88% of broiler flocks used ionophores and 45% used chemical coccidiostats. There were six different ionophores (lasalocid [LAS], maduramicin [MAD], monensin [MON], narasin [NAR] narasin-nicarbazin [NARNIC], salinomycin [SAL]) and 6 different chemical coccidiostats (clopidol [CLO], decoquinoate [DEC], diclazuril [DIC], nicarbazine [NIC], robenidine [ROB] and zoaline [ZOA]) used; temporal variations were observed for certain drugs. Overall, 72% (365/509) of broiler flocks were under a shuttle/dual control program (>1 drug/cycle) and the remaining flocks used a continuous/straight program (1 drug/cycle) (18%) or did not use any drugs (10%) but were vaccinated against coccidiosis (raised without antibiotics/organic flocks). The most common practice under a shuttle/dual control program involved the sequential (no specific order) administration of the combination coccidiostat NARNIC, then either NAR (62 flocks) or SAL (33 flocks). There were at least 61 other drug combinations used in a shuttle program. Under a continuous/straight program, the most common practice involved the use of SAL (49 flocks) and MON (20 flocks) as the sole coccidiostat administered throughout the growing period.

For NE prevention, there were five different antimicrobials reported (tylosin [TYL], penicillin [PEN], virginiamycin [VIR], bacitracin [BAC] and avilamycin [AVI]). Most broiler flocks (52%, 267/509) used only one antimicrobial throughout the cycle; the most frequently used were BAC (166 flocks), VIR (52 flocks) and AVI (30 flocks). When the NE program involved the sequential administration of ≥ 2 antimicrobials (36%, 183/509) during the grow-out period, the most frequently used were a BAC-AVI program (52 flocks), a BAC-VIR program (25 flocks) and a PEN-VIR program (20 flocks). At least 18 other NE programs involving ≥ 2 antimicrobials were reported.

Turkeys. There were fewer types of coccidiostats reported compared to broiler chickens (ionophores: LAS, MAD,MON; chemical coccidiostats: CLO, DIC, ROB, ZOA). Unlike in broilers (largely under shuttle programs), the vast majority of the turkey flocks used a continuous control program (76%, 122/160); the most frequently used were MON (66 flocks) and LAS (46 flocks). Under a shuttle program, the most common practice involved the sequential administration of MAD then DIC during the grow-out period. There were only eight other shuttle programs utilized in turkey flocks, fewer than in broilers.

For NE prevention in turkeys, there were five antimicrobials used (TYL, PEN, VIR, BAC, and oxytetracycline [OXY]). Most flocks (73%, 116/160) used only one antimicrobial throughout the growing period; the most commonly used were VIR (56 flocks) and BAC (55). When the NE program involved the sequential administration of ≥ 2 antimicrobials (11%, 17/160) during the grow-out period, the only three antimicrobial programs reported were the successive (not in particular order) use of PEN then BMD (7 flocks), VIR then BMD (7 flocks) and PEN then TYL(3 flocks).

Flock health status. During the study timeframe, the number of producers/veterinarians that reported their clinical assessment of NE and coccidiosis status in broiler chickens (i.e. likely positive or positive based on at least one of: clinical signs, post-mortem or laboratory diagnosis) was 13% and 4%, respectively. In turkeys, NE and coccidiosis diagnoses were relatively lower at 2% and 3%, respectively. During outbreaks of enteric diseases, diagnosed flocks were treated via water with sulfonamides (coccidiosis) and penicillins (NE).

Risk factor analysis (broiler chickens only). Using a logistic regression model, preliminary analysis indicated that the following operational/farm factors were significantly associated with an increased odds of having a coccidiosis positive status: antimicrobial free status, no downtime, barn population (<25,000) and stocking density. The following operational/farm factors were significantly associated with decreased odds of having a coccidiosis positive status: downtime, barn population (>25,000), and region (West). Salmonellosis status, hypothesized to play a role in gut integrity had no effect although this may have been due to the small number of samples per flock. Using a negative binomial model,

preliminary analysis indicated that the following operational/farm factors were significantly associated with higher mortality counts per 1000 chicken days at risk: region, barn population, coccidiosis and airsacculitis status, antimicrobial free status, and use of injectable antimicrobials at the hatchery (i.e. poor quality chicks may have been anticipated due to breeder flock issues or other operational factors). The following operational/farm factors were significantly associated with decreased mortality counts per 1000 chicken days at risk: season (flocks reared during the spring, summer and fall), cleaning and disinfection between flocks and vaccination against infectious bursal disease (an immunosuppresive viral disease).

CONCLUSIONS

With upcoming Canadian federal legislation and industry-led initiatives toward AMU stewardship as well as to address changing consumer needs (i.e. increased demand for meat raised under antimicrobial-free production), AMU practices, particularly for managing both NE and coccidiosis, may change. Understanding the impact of farm practices and current AMU and vaccination approaches shuld help manage NE and coccidiosis in the field and will potentially facilitate the reduction of AMUin alignment with national and global initiatives aimed at preserving the efficacy of antimicrobials and reducing AMR risks.

(Full-length article will be submitted to *Avian Diseases*.)

REFERENCES

1. PHAC 2015. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) Annual Report 2013 - Chapter 1. Design and Methods. [cited 26 January 2016]. Available from: http://publications.gc.ca/collections/collection_2015/aspc-phac/HP2-4-2013-1-eng.pdf.

2. EMA, Veterinary Medicines Division 2015. Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2013, Fifth ESVAC Report [cited 26 January 2016]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2015/10/WC500195687.pdf.

CHANGES IN THE PREVALENCE OF RESISTANCE IN *E. COLI, SALMONELLA* AND *CAMPYLOBACTER* FROM BROILER CHICKENS AND ANTIMICROBIAL USE ON-FARM; SURVEILLANCE RESULTS, 2013-2016

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SUMMARY

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) has as one of its objectives, to describe the prevalence and antimicrobial resistance (AMR) of select enteric bacteria in broiler chicken flocks. In May 2014, the Canadian poultry industry implemented a voluntary removal of the preventive use of antimicrobials belonging to the Veterinary Drugs Directorate (VDD) of Health Canada, Category I (very high importance to human medicine). For the broiler chicken industry this decision primarily affected the use of the antimicrobials ceftiofur (a 3rd generation cephalosporin [3 rd GC]) and enrofloxacin (a fluoroquinolone). Shortly after this, CIPARS detected a decrease in resistance to ceftriaxone, a 3rd GC antimicrobial, in chicken *Salmonella* and *E. coli* isolates from farm samples. The decrease in the frequency of ceftriaxone resistance in *Salmonella* and *E. coli* mirrors an observed decrease in ceftiofur use at the hatcheries. For two consecutive years (2015 and 2016), there have been no producers reporting the use of ceftiofur at the hatcheries. During the same timeframe, gentamicin resistance trended upwards and appears to correlate with the reported use of gentamicin and lincomycin-spectinomycin in chicks or hatching eggs. Resistance in *E. coli* to trimethoprim-sulfonamides also increased. This drug combination was reported as used in broiler chickens via feed to treat systemic and localized infections (respiratory). Resistance to ciprofloxacin in *Campylobacter* from broiler chickens persisted despite no reported use of enrofloxacin. The decrease in resistance to Category 1 antimicrobials in *E. coli* and *Salmonella* suggests that the industry-led AMU policy change is having the desired goal of reducing resistance and use of antimicrobials of very high importance to human medicine but might also have resulted in changes in antimicrobial use for treating infections in young chicks. The persistency of ciprofloxacin-resistant *Campylobacter* requires further investigation. This presentation highlights the importance of surveillance in measuring the impact of interventions to reduce AMU and AMR.

MATERIALS AND METHODS

For each enrolled farm, four pooled fresh cecal samples (equivalent to 10 individual droppings) from the four quadrants of the barn were collected from the barn floor from birds ≥30 days of age. Approximately half of the flocks were also visited at placement for sampling of chick paper pads and the environment. Farm-level AMR results were adjusted for clustering to account for multiple samples collected per farm.

Details regarding methods used for sample collection, culture, and antimicrobial susceptibility testing are available in the CIPARS annual reports (1).

RESULTS AND DISCUSSIONS

Bacterial recovery rates across programs

Salmonella. Overall, recovery rates have fluctuated over the last 4 years (44% to 59%) but between 2015 and 2016, the recovery rate at preharvest decreased by 23% (2015: 59%; 2016: 36%). Serovar distribution in each province also changed from year to year but the top three sevovars remain consistent. In 2016, the top three serovars detected were Kentucky (36.8%, 92/250), Enteritidis (16%, 40/250) and Heidelberg (11%, 28/250). The overall decrease in recovery on-farm in 2016 may be an indication of the impact of farm-level interventions such as vaccination in breeders and ongoing enhancements to the industry food safety programs; however, despite these efforts, *S.* Enteritidis was still detected in all regions/provinces included in the surveillance.

Escherichia coli. The overall recovery rate has remained stable over the last four years (99%).

Campylobacter. The recovery rate at preharvest sampling fluctuated over time and ranged from 17% to 22% over the last four years. Between 2015 and 2016, it decreased by 13% (2015: 59%; 2016: 46%). The majority of the isolates were *C. jejuni* (83%, 77/93).

Antimicrobial resistance

Chick placement sampling

Salmonella. Overall, resistance to ceftriaxone decreased from 29% to 3% between 2013 and 2016. Gentamicin modestly increased from 0% to 6% during the same timeframe.

Escherichia coli. Overall, resistance to ceftriaxone significantly decreased from 39% to 18% between 2013 and 2016. Gentamicin resistance has been somewhat stable over the last four years, although a modest decrease from 39 % to 28% was noted between 2015 and 2016.

Preharvest sampling stage

Salmonella. Resistance to ceftriaxone decreased significantly over the last four years from 22% to 7%. Over the same timeframe, resistance to gentamicin modestly increased from 0.4% to 3%. In 2016, other than ceftriaxone, gentamicin, and ampicillin, no remarkable increases in resistance to any other antimicrobials (including: nalidixic acid, streptomycin, tetracycline, trimethoprim-sulfonamide) tested were observed. No isolates exhibited resistance to meropenem, a carbapenem antimicrobial.

E. coli. Similar to *Salmonella*, resistance to ceftriaxone decreased significantly over the last four years from 32% to 9%. During the same timeframe, resistance to gentamicin increased significantly from 13% to 21%. In 2016, other than resistance to ceftriaxone, gentamicin, and ampicillin, no remarkable increases in resistance to any other antimicrobials (including: streptomycin, tetracycline, trimethoprim-sulfonamide) tested were observed. The decrease in ceftriaxone resistant *E. coli* reflected the reported decrease in the use of ceftiofur at the hatcheries more closely than the *Salmonella* data. No isolates exhibited resistance to meropenem.

Campylobacter. Resistance to ciprofloxacin continued to be detected between 2015 (16%) and 2016 (135). Between 2013 and 2016, tetracycline resistance significantly decreased from 59% to 22%.

Antimicrobial use. Antimicrobials relevant to the AMR findings described above were used at the hatchery and on-farm. Twenty three percent (31/136) of broiler producers reported that the chicks delivered to their barns were medicated at the hatchery. This reported use at the hatchery decreased from 39% in 2015. There have been no producers reporting ceftiofur use at the hatchery for two consecutive years (2015 and 2016). Gentamicin use significantly decreased from 2015 to 2016 from 10% to 3% and lincomycin-spectinomycin use modestly decreased from 30% to 20% during the same timeframe. At the farm, trimethoprim-sulfadiazine administered via feed was reported but the trend in use appears to be decreasing (11% in 2015; 8%: 2016) and it was used only in two provinces/regions.

CONCLUSIONS

The industry-led intervention to eliminate the preventive use of VDD Category I antimicrobials appears to have resulted in a decrease in resistance to ceftriaxone among generic *E. coli* and *Salmonella* isolated from broiler chickens at the farm. CIPARS data suggest that the change in AMU policy resulted in a switch to other AMU options for managing ongoing flock health issues (i.e. gentamicin and lincomycin-spectinomycin for treating neonatal infections). The persistence of ciprofloxacin resistance in *Campylobacter* may be due to the ability of ciprofloxacin-resistant *Campylobacter* to outcompete ciprofloxacin-susceptible *Campylobacter* in the absence of selection pressure (2) or AMU that were not captured in our surveillance timeframe. CIPARS will continue to collect farm-level information to monitor trends, better understand risk factors that may impact AMU/AMR, and to assess the impact of other voluntary industry initiatives and federal legislative changes on AMU and AMR.

REFERENCES

1. PHAC 2015. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) Annual Report 2013 - Chapter 1. Design and Methods. [cited 26 January 2016]. Available from: http://publications.gc.ca/collections/collection_2015/aspc-phac/HP2-4-2013-1-eng.pdf.

2. Luo N, Sahin O, Lin J, et al. Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc Natl Acad Sci USA. 102: 541-546. 2005

CHARACTERIZATION OF INFECTIOUS BRONCHITIS VIRUS INFECTION IN WESTERN CANADA LAYER FLOCKS AFFECTED WITH SHELL-LESS EGG SYNDROME

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ABSTRACT

Shell-less egg syndrome (SES) is a condition of unknown etiology, which occurs as sporadic outbreaks in the Western Canada layer operations for the last three years causing economic losses to the layer industry. SES is likely to be caused by a "variant" infectious bronchitis virus (IBV). This suspicion is based on the ability of IBV to cause egg production problems and observation of sub-optimal levels of protective antibody titers in the affected flocks. Therefore, the current study aims at investigating whether SES is associated with IBV variants. So far, 602 tissue samples from 52 flocks were screened for IBV using an optimized real-time polymerase chain reaction (real-time PCR) assay targeting a fragment of the IBV nucleoprotein gene. Seventy-nine out of 602 samples were positive for the IBV and many of them were from the acutely-affected flocks with SES. Full-length nucleotide sequence of the hypervariable spike protein-1 gene (S1) sequence was obtained from six and the partial S1 sequence was obtained from 13 real-time PCR positive samples. We chose two of these isolates based on the full-length S1gene sequence to evaluate the pathogenic significance and tissue tropism. Both isolates replicated in kidney and the reproductive tract *in vivo*. An experimental study is underway to reproduce the SES from selected two isolates from this study.

INTRODUCTION

Field observations have shown an increased incidence of transient shell-less egg production in the layer operations in Western Canadian provinces for the last 2-3 years. This occurs as sporadic outbreaks in layers of varying age at lay and as a common characteristic of this condition, affected flocks generally show a drop-in egg production in addition to the production of shell-less eggs. This condition is called as "shell-less egg syndrome" (SES) and it is likely to be caused by a variant strain of the infectious bronchitis virus (IBV) since some strains of IBV target shell gland of the reproductive tract where the shell of the egg is formed. IBV is known to cause egg-shell abnormalities in layers (9) and there are records showing altered tissue tropism in newly emergent IBV variants (3, 6, 7, 10). Furthermore, field observations such as improper IBV vaccination practices adopted in the farms, sub-optimal protective IBV antibody levels detected in the affected flocks, and the non-specific histopathological lesions of the shell-gland, which are suggestive of a viral infection strongly support our speculation. IBV is a well-known host-specific respiratory pathogen, which belongs to the family Coronaviridae. However, IBV can replicate in various body compartments and organs (2, 8) including the shell gland causing various eggshell abnormalities, reduced egg production by 3-50% and internal egg quality depending on the age at lay and the strain of the virus (1, 4).

This study aimed at investigating whether SES observed in layer flocks in Western Canada is associated with a variant IBV. Secondly, we inspected the virulence of selected IBV isolates in experimental chickens. Finally, a pilot experiment will be carried out in an attempt of reproducing the SES in experimental birds with variant IBV isolates and investigating associated pathology in the oviduct causing SES.

MATERIALS AND METHODS

Screening of samples for infectious bronchitis virus (IBV) and molecular characterization. Samples were collected from layer flocks with the history of SES raised in Saskatchewan and Alberta. Viral RNA was extracted using a Trizol® based method and an optimized real-time polymerase chain reaction (PCR) assay was used to amplify 200 base pairs (bp)-sized fragment of the IBV nucleoprotein (N) gene (5). Further, ten real-time PCR positive samples

were 5-7 times passaged on embryo-day 9-11 chicken eggs to amplify the virus. The hypervariable spike protein 1 gene (S1) of the IBV (1760 bp) isolated from the allantoic fluid was amplified using conventional reverse-transcriptase PCR. A short fragment (620 bp) of the IBV S1 gene was amplified from the remainder of the real-time positive samples. Reverse-transcriptase positive samples were direct Sanger-sequenced and resulting nucleotide sequences were compared with the reference IBV strains and serotypes to determine the genetic variation of IBV isolates of this study.

Determination of the virulence and the tissue tropism of the selected IBV isolates. Day-6 old specific-pathogen-free chickens were infected with two of the selected IBV isolates from this study (n=6 in each infected and uninfected-control groups) and observed for clinical picture and oro-pharyngeal and cloacal swab samples were taken in addition to terminal tissue samples taken at four and 14 days-post-infection (dpi). The IBV genome load was determined from both swab and tissue samples and immunohistochemistry and histopathology were carried out on both tracheal and lung tissues.

Experimental reproduction of SES using the selected IBV isolates. Twenty-two-week-old SPF Leghorn layers will be inoculated with above two IBV isolates as a pilot study in an attempt of reproducing the SES under experimental conditions. Egg production pattern will be observed for 12 dpi and tracheal and cloacal swab samples will be taken at timely intervals. Gross and histopathological changes will be evaluated on necropsies of birds upon termination of the experiment at 12 dpi and the IBV genome load will be determined on swab and tissue samples.

RESULTS AND DISCUSSION

So far, 602 tissue samples from 52 flocks were screened for IBV using the optimized real-time polymerase chain reaction (real-time PCR) assay. Seventy-nine out of 602 samples were positive for the IBV. Full-length nucleotide sequence of the hypervariable spike protein-1 gene (S1) sequence was obtained from six and the partial S1 sequence was obtained from 13 real-time PCR positive samples. Based on the phylogram drawn using the IBV S1 short fragment nucleotide sequence, our IBV isolates were clustered with the Massachusetts and Connecticut serotypes while one isolate was clustered in between these serotypes. We chose two of those isolates (isolate 1 and 2) based on the full-length S1 gene sequence to evaluate the pathogenic significance and tissue tropism. Both isolates replicated in kidney and the reproductive tract *in-vivo* as demonstrated by the presence of the IBV genome load.

We noticed that 5-7 embryonated-egg passages were required to increase the detection limits of the IBV isolated from the field samples via reverse-transcriptase PCR assay of the full-length S1 gene. Therefore, we attempted to amplify a short fragment of the S1 gene directly from the real-time PCR positive field isolates. This method was successful for a portion of samples with lower real-time PCR cycle-threshold values in our initial screening assay. Owing to this reason we got only 19 out of 79 real-time positive samples successfully sequenced for the molecular characterization. Currently, an experiment is being undertaken to reproduce SES with the IBV variants isolated from this study.

ACKNOWLEDGEMENTS

We acknowledge funding from Natural Sciences and Engineering Council of Canada (NSERC) through NSERC-discovery (RT736458) and NSERC-research tools and instrument (RTI) grants and Saskatchewan Agriculture Fund (20140185).

(The full-length article will be published in an appropriate journal.)

REFERENCES

1. Cavanagh, D. Coronavirus avian infectious bronchitis virus. Vet Res 38:281-297. 2007.

2. Cook, J. K., S. J. Orbell, M. A. Woods, and M. B. Huggins. Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Pathology: Journal of the W.V.P.A 28:477-485. 1999.

3. Cumming, R. B. Studies on Australian infectious bronchitis virus. IV. Apparent farm-to-farm airborne transmission of infectious bronchitis virus. Avian Diseases 14:191-195. 1970.

4. Ignjatovic, J., and S. Sapats. Avian infectious bronchitis virus. Revue Scientifique et technique (International Office of Epizootics) 19:493-508. 2000.

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

6. Kingham, B. F., C. L. Keeler, W. A. Nix, B. S. Ladman, and J. Gelb. Identification of Avian Infectious Bronchitis Virus by Direct Automated Cycle Sequencing of the S-1 Gene. Avian Diseases 44:325-335. 2000.

7. Liu, S., X. Zhang, Y. Wang, C. Li, Q. Liu, Z. Han, Q. Zhang, X. Kong, and G. Tong. Evaluation of

the protection conferred by commercial vaccines and attenuated heterologous isolates in China against the CK/CH/LDL/97I strain of infectious bronchitis coronavirus. The Veterinary Journal 179:130-136. 2009.

8. Seo, S. H., and E. W. Collisson. Specific cytotoxic T lymphocytes are involved in vivo clearance of infectious bronchitis virus. Journal of Virology 71:5173-5177. 1997.

9. Sevoian, M., and P. Levine. Effects of infectious bronchitis on the reproductive tracts, egg production, and egg quality of laying chickens. Avian Diseases 1:136-164. 1957.

10. Song, C. S., Y. J. Lee, J. H. Kim, H. W. Sung, C. W. Lee, Y. Izumiya, T. Miyazawa, H. K. Jang, and T. Mikami. Epidemiological classification of infectious bronchitis virus isolated in Korea between 1986 and 1997. Avian Pathology 27:409-416. 1998.

INVESTIGATION OF INTERLEUKIN (IL)-1B AND MACROPHAGE RESPONSES IN THE RESPIRATORY TRACTS OF CHICKENS FOLLOWING INFECTIOUS BRONCHITIS VIRUS INFECTION

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ABSTRACT

Infectious bronchitis is one of the economically important viral diseases in chickens worldwide, which cannot be controlled effectively by vaccination. Therefore, we believe that innate immune mechanisms of chickens should be explored to seek potential value for the control of this disease. As mediators of the early innate host response, macrophages and cytokines, such as interleukin (IL)-1β, are critical components as shown in other host-virus infection models. We studied the association of IL-1β, originating from avian macrophages and IBV infection in the respiratory tract of specific-pathogen-free chickens. We observed elevated levels of IL-1β and increased recruitment of macrophages in the respiratory tract of IBV infected chickens compared to uninfected control chicks. Further, IBV genome load of the infected tissues showed a correlation with these two innate immune mediators. In conclusion, the present study demonstrates that recruitment of macrophages and the production of IL-1β originating from macrophages, as well as other sources, occurs following IBV infection in the respiratory tract suggesting potential roles of these mediators in combating the initial IBV replication at the route of entry.

INTRODUCTION

Infectious bronchitis is an acute respiratory infection in chickens caused by the infectious bronchitis virus (IBV). IBV is primarily controlled by vaccination and emergence of heterogeneous new variants of IBV, has led to the deceased efficacy of the currently used vaccines. Therefore, it is essential to explore alternative control options, such as harnessing chickens' own innate immune functions. IL-1β is one of the pro-inflammatory cytokines, which is produced by avian macrophages and the epithelial cells during virus infection (2). IL-1β works as a chemotactic factor, whereby blood monocytes move to the injured site and differentiate into tissue macrophages. This initiates the cellular immune responses including recruitment of macrophages to the injured sites (6). Macrophages are considered as critical cells of the innate immune response, are produced in the bone marrow and have a distinct function of phagocytosis of microbes (5, 7). Additionally, the macrophages are a source of pro-inflammatory cytokines such as IL-1β and act as antigen presenting cells to guide the initiation of antigen-specific adaptive immune responses. Also, macrophages are a source of inducible nitric oxide syntheses (iNOS) (4), which catalyze the production of nitric oxide (NO) in order to induce antiviral responses (1, 3). Previous studies have shown that in both the trachea and lungs, IL-1β mRNA expressions were increased upon IBV infection in chickens(4). However, it is not yet known whether protein expressions of IL-1β originated from macrophages or any other type of cells are associated with IBV infections in the respiratory tract of chickens. In this study, we used an established IBV experimental challenge to investigate the kinetics of recruitment of macrophages and the expression of IL-1β in both the trachea and lungs of chickens.

MATERIALS AND METHODS

Twenty 6-day old chickens were infected intratracheally with 2.75×10^4 plaque forming units (PFUs) of the IBV M41 strain and another 20 birds served as uninfected controls. Four IBV-infected and four control chickens were euthanized at 1, 2, 3, 5 and 7 days post-infection (dpi) and trachea and lungs were collected for genome load quantification, immunohistochemistry, and histopathology. The IBV genome loads of trachea and lungs were estimated using an optimized quantitative PCR (qPCR) protocol targeting the IBV nucleoprotein gene (N). Frozen sections of trachea and lung tissues were immunofluorescence-stained for both macrophage and

IL-1β antigens consecutively. Those areas stained with each type and combined fluorochrome-signal were expressed as a percentage of the total area of each tissue section using a semi-quantitative method. To identify group differences, either student's t-test or one-way analysis of variance (ANOVA) test followed by Tukey's test were performed using Prism 5 software (GraphPad, La Jolla, CA, USA). The statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Histopathological examination of both trachea and lung at 1, 2, 3, 5, 7 dpi showed lesions suggestive of infectious bronchitis. We observed different patterns of recruitment of macrophages and the expression of IL-1β between the trachea and lungs of IBV infected chickens. Further, patterns of macrophage recruitment and the expression of IL-1β followed the patterns of IBV genome load in both the trachea and lungs. The IBV genome loads and macrophage numbers peaked at 5 dpi in both the tracheal and lung tissues. But only in the trachea of IBV infected chickens, macrophage numbers declined at 7 dpi which coincided with a significant decrease in IBV genome load. The expression of IL-1β, in the trachea of IBV infected chickens, followed the patterns of IBV genome load and macrophages recruitment. However, the expression pattern of IL-1β in the lungs of IBV infected chickens was different from that observed in the trachea and was increasing until seven dpi. The IL-1β positive macrophages peaked at five and seven dpi in the trachea and in the lungs of IBV infected chickens, respectively, which is very similar to the pattern of IBV genome loads observed in these tissues. In our study, we evidenced that the IBV genome loads declined in the trachea and showing a declining trend in lungs by seven dpi and this decrease may potentially be attributable to the development of adaptive immune responses against IBV infection. Also, the current study witnessed the expression of IL-1β by macrophages towards the end of the study in both the trachea and lungs. This suggests that although macrophages were recruited to the respiratory tract early phase of the IBV infection, production of IL-1β by macrophages was prominent towards the latter part of the infection. The peak IL-1β production by macrophages was associated with decreasing IBV genome load in the trachea. Interestingly, we found a significant positive correlation between IBV genome load and macrophage recruitment only in the lungs. In the uninfected lungs, higher levels of expression of IL-1β was observed compared to the corresponding tracheas and we believe this difference potentially is due to the difference in the amount of mucosal tissues between trachea and lungs.

In conclusion, we found increased IL-1β expression in both tracheal and lung tissues in response to experimental IBV M41 infection. Macrophage recruitment also showed a similar increase during IBV infection, but these cells were not a significant source of IL-1β until towards the latter part of the IBV infection in both the trachea and lungs. It is possible that the source of the expression of IL-1β during the initial stage of IBV infection could be cells other than macrophages. We found a significant association between the IBV genome load and macrophage recruitment only in lungs and the expression of IL-1β response in both trachea and lungs suggesting potential roles of macrophages in the host responses to IBV infection. However, further studies are warranted to elucidate whether macrophages and IL-1β help in limiting the IBV replication in the respiratory tract and to observe whether other immune mediators are involved in the antiviral response against the IBV infection.

ACKNOWLEDGEMENTS

We acknowledge funding from Natural Sciences and Engineering Council of Canada (NSERC) through NSERC-discovery (RT736458) and NSERC-research tools and instrument (RTI) grants and Saskatchewan Agriculture Fund (20140185).

(The full-length article has been accepted to be published in *Veterinary Microbiology*.)

REFERENCES

1. Abdul-Cader, M. S., A. Amarasinghe, and M. F. Abdul-Careem. Activation of toll-like receptor signaling pathways leading to nitric oxide-mediated antiviral responses. Archives of Virology 161:2075-2086. 2016.

2. Babcock, A. A., H. Toft-Hansen, and T. Owens. Signaling through MyD88 Regulates Leukocyte Recruitment after Brain Injury. The Journal of Immunology 181:6481-6490. 2008.

3. Haddadi, S., D.-S. Kim, H. Jasmine, F. van der Meer, M. Czub, and M. F. Abdul-Careem. Induction of Toll-like receptor 4 signaling in avian macrophages inhibits infectious laryngotracheitis virus replication in a nitric oxide-dependent way. Veterinary Immunology and Immunopathology 155:270-275. 2013.

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

5. Mast, J., B. M. Goddeeris, K. Peeters, F. Vandesande, and L. R. Berghman. Characterisation of chicken monocytes, macrophages and interdigitating cells by the monoclonal antibody KUL01. Veterinary Immunology and Immunopathology 61:343-357. 1998.

6. Qureshi, M. A., C. L. Heggen, and I. Hussain. Avian macrophage: effector functions in health and disease. Developmental & Comparative Immunology 24:103-119. 2000.

7. Reddy, V. R. A. P., I. Trus, L. M. B. Desmarets, Y. Li, S. Theuns, and H. J. Nauwynck. Productive replication of nephropathogenic infectious bronchitis virus in peripheral blood monocytic cells, a strategy for viral dissemination and kidney infection in chickens. Veterinary Research 47:70. 2016.

WHOLE GENOME SEQUENCING AND ANALYSIS OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) ISOLATES

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SUMMARY

Infectious bursal disease virus (IBDV) is an RNA virus with segmented double stranded genome. The virus mainly affects the bursa of Fabricius causing severe immunosuppressive disease. In this study, we isolated five different variant strains of IBDV from samples collected from poultry farms in Saskatchewan, Canada. The virus isolates cause characteristic gross and histopathologic lesions of IBD. The viruses appear to be genetically different from vaccine strains based on phylogenetic analysis of segment A and B gene sequences. Recombination events were detected between segment A sequences of a couple of our isolates and reference sequences retrieved from the Genbank. Of the viral proteins, VP2 was the most variable among the isolates. Analyses of domains of the VP2 protein of each virus indicate that the most variable domain was the loop regions of the P (projection) domain. Furthermore, based on structural modelling, a difference in the structure of the loop regions of the P domain of VP2 proteins was observed with a single amino acid substitution enough to modify the loops.

INTRODUCTION

Infectious bursal disease (IBD) also called Gumboro disease is caused by IBD virus which is a member of the family *Birnaviridae*. It is an economically important immunosuppressive disease of poultry. IBDV is a non-enveloped virus containing two segments of double stranded RNA (segment A and B) (1). Segment A encodes viral protein (VP)2, VP3 and VP4 whereas segment B encodes VP1, and VP5 (24). VP2 is the major structural protein responsible for inducing neutralizing antibodies (2). Within the coding region of VP2 a "hyper-variable domain" exists which contains two major hydrophilic regions (2). Substitution mutations in these areas contribute to antigenic drifts occurring in the virus which may result to the emergence of "variant strains." The emergence and distribution of new variant strains can occur in a particular geographic area (3). But intensive trading can easily lead to spreading of the new viruses around the world (4). Since 2007, variant strains of IBDV have been increasingly isolated from "problem broiler flocks" across major Canadian provinces (5). Hence, the objective of this study was to isolate and characterize variant IBDVs at the full genome level.

MATERIALS AND METHODS

RT-PCR, sequencing and sequence analysis. RNA was extracted from bursal homogenates using the RNeasy kit (Qiagen, USA) as per the manufacturer's instructions. The RT-PCR was performed using One-Step RT-PCR Kit (Qiagen, USA). A set of specific primers were designed to amplify segment A and B genes of each IBDV isolate. The fragments were sequenced (Macrogen Inc., Korea) and each genome was assembled using ContigExpress module of Vector NTI advance 9 software (Invitrogen). Mapping of protein coding regions of the genomic segments were performed based on previously reported full-length sequences of IBDV. A phylogenetic analysis was performed using Geneious 9.1.5 software by the neighbor-joining method with 1,000 bootstrap replicates. The structure of the VP2 protein of each virus was modelled using MODELLER software (6) and visualized by PyMOL2.0 software (The PyMol Molecular Graphics System, Schrodinger, LLC). Recombination events were analyzed using Recombination Detection Program 4 (RDP4) (7).

RESULTS AND DISCUSSION

Phylogenetically, the complete genome sequences of Segement A and B of the isolates were closely related to variant IBDV strains recovered from GenBank. Of all the five gene products of each virus, a high variability was observed in the VP2 region. This

has been previously described by several researchers (2). Within the VP2 protein of the isolates, nearly 40% of the amino acid differences occur in the loop regions indicating that these are the sites where antigenic drift occur most frequently. This antigenic drift has contributed to the emergence of variant IBDV and the increased incidence of subclinical immune suppression in broilers despite usage of commercial vaccines in Canada. Based on structural modelling, the amino acid substitutions in each loop region of VP2 protein were enough to change the structure of the loop. Moreover, analysis of segment A gene sequences of IBDV retrieved from GenBank for recombination events using RDP4 suggest that new variant strains can emerge through recombination between different strains. The results also indicate that a couple of the Canadian variant IBDV isolates possibly emerged as a result of recombination events between different strains of IBDV. Moreover, the failure in the success of commercial IBD vaccines in preventing disease in Canada might be associated with antigenic escape by these variant viruses due to antigenic drift in the loop region of the VP2 protein.

REFERENCES

1. Mundt, E., J. Beyer, and H. Muller Identification of a novel viral protein in infectious bursal disease virus –infected cells. Journal of General Virology 76:437-443. 1995.

2. Eterradossi, N., D. Toquin, G. Rivallan, and M. Guittet Modified activity of a VP2-located neutralizing epitope on various vaccine, pathogenic and hypervirulent strains of infectious bursal disease virus. Archives of Virology 142:255-270. 1997

3. Jackwood, D.J., and S.T. Stoute Molecular Evidence for a Geographically Restricted Population of Infectious Bursal Disease Viruses. Avian Diseases 57:57-64. 2012.

4. Hernández, M., G. Tomás, A. Marandino, G. Iraola, L. Maya, N. Mattion, D. Hernández, P. Villegas, A. Banda, Y. Panzera, and R. Pérez Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage. Avian Pathology:1-27. 2015.

5. Ojkic, D., E. Martin, J. Swinton, B. Binnington, and M. Brash Genotyping of Canadian field strains of infectious bursal disease virus. Avian Pathology 36:427-U118. 2007.

6. Eswar, N., Eramian, D., Webb, B., Shen, M. Y. and A Sali Protein structure modeling with MODELLER. Methods Mol Biol. 426:145–159. 2008.

7. Martin, P.D., Murrell, B., Golden, M., Khoosal, A. and B Muhire RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evolution, 1(1): vev003. 2015.

GLOBAL RESEARCH ENSUING IN AN INVENTION FOR COMPREHENSIVE CONTROL OF COCCIDIOSIS IN POULTRY

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ABSTRACT

A global data of 13 experiments, accomplished collectively in nine countries, aimed at replacement of synthetic poultry coccidiostats by an invented comprehensive dual approach of decontaminating poultry barns by an invented wide spectrum disinfectant (WSD) and intermittent supplementation of drinking water with natural essential oil blend emulsified in water extract (EOBWE) of plants. The first nine trials were concluded in isolation unit facilities and laboratories, while the other four were field trials. These first nine trials had different objectives including, studying the protection against coccidiosis by intermittent or continuous administration of EOBWE in drinking water against controlled challenge by sporulated oocytes of *Eimeria* spp., administered intra-esophageally or through contaminated floors of rearing pens. Another objective studied the effect of different concentrations of EOBWE and WSD on lysis of *Eimeria* oocytes and inhibition of wide spectrum of poultry microorganisms, while other objective compared the control of coccidiosis in broilers by the invented dual approach of applying WSD and EOBWE versus the application of classical disinfectants and synthetic coccidiostats. The first three field trials compared the dual intervention by classical disinfectants and synthetic coccidiostats versus the invented intervention by WSD and EOBWE against field challenge of broilers by *Eimeria* spp. The fourth trial compared the impact of synthetic coccidiostat alone versus concurrent administration of both the synthetic coccidiostat and the EOBWE on protection of broilers against field challenge by *Eimeria acervulina* spp. The compiled data of this global research led to comprehensive control of poultry coccidiosis, manifested in significant reduction of oocytes output and its associated *Eimeria* lesions, and consistent improvement of the chicken performance.

INTRODUCTION

Coccidiosis is the most economic protozoan disease, affecting poultry industry worldwide, resulting in an annual loss of more than four billion US dollars (7). The continuous supplementation of coccidiostats in feed did result in the emergence of drug-resistant *Eimeria* strains (6), and sporadic residuals of drugs in poultry products, creating a concern in consumers (4). It is documented that the synergistic multiplicity of active ingredients, present in disinfectants, essential oils and water extracts of plants, makes it difficult for bacteria, viruses, yeasts, and protozoa to develop a resistance to it (2, 5). The aim of this work is built on the hypothesis of dual intervention approach by an invented WSD, with multiplicity of active ingredients that inactivated a wide spectrum of economic poultry pathogens (2), and intermittent supplementation of poultry-drinking water by natural EOBWE of plants, also with multiplicity of active ingredients, which could result in a comprehensive control of poultry coccidiosis.

MATERIALS AND METHODS

The materials and methods of the compiled 13 experiments aimed at evaluation of a new method for control of coccidiosis in poultry by dual intervention with an invented WSD to decontaminate the surfaces of rearing area, followed by intermittent supplementation of drinking water with invented EOBWE. The invented WSD is a mixture of phenol derivatives, organic solvent, organic acids, inorganic acids and an anionic surfactant, while the invented EOBWE is a mixture of essential oils, emulsified in water extracts of herbs (3).

Experiment #1, performed at Leipzig University, Germany, studied the % lysis of *E. tenella*-sporulated oocytes (25,000 oocytes/mL) by contact with 3% and 4% dilutions of invented WSD for periods of 30 to 120 minutes vs. control oocytes deprived of contact with WSD (1).

Experiment #2, performed also at Leipzig University, Germany, studied the infectivity in day-old chicks (N = 10/treatment) by sporulated oocytes of *E. tenella* (2000 oocytes/chick) that were previously contacted with 3 % WSD for 120 min (Treatment #1) vs. infectivity by non-contacted oocytes with WSD (Treatment #2), following the guidelines of the German Veterinary Medicine Society, 2007 (1), while controls were deprived of any challenge (Treatment #3).

Experiment #3, performed by Opticon CO.-Switzerland and American University of Beirut- AUB, examined the susceptibility of poultry-associated bacteria and viruses upon contact with 3% dilution of invented WSD. The tested bacteria were *E. coli*, *Streptococcus* spp., *Salmonella* Enteritidis, and *Proteus mirabilis*, while the tested viruses were Newcastle disease virus (lentogenic) and avian influenza (H9N2). The bacterial count before contact was adjusted to $2.0x10^7$ CFU/ mL, while the viral HA units before contact was set at HA units of 4.0. Each test was performed in triplicate.

Experiment #4, performed at AUB, determined the needed contact time between oocytes and different dilutions of EOBWE for obtaining a maximum lysis. The total oocyst number in each test was 25,000 /mL of equivalent number of 8 *Eimeria* spp. (*E. acervulina, E. brunetti, E. hagani, E. maxima, E. mivati, E. necatrix, E. praecox, and E. tenella*). The developed EOBWE was used in four different dilutions of 0.005, 0.010, 0.020, and 0.100 %. The contact times were 30, 60, 90, 120, and 180 min. Control oocytes count of 2500/mL were kept in contact with saline. All tests were accomplished in duplicate.

Experiment #5, performed by Opticon Co., Switzerland and AUB, aimed at evaluation of the susceptibility of poultry-associated bacteria and viruses to invented EOBWE, using the same above protocol, presented under Experiment #3, except that each test was accomplished in triplicate.

Experiment #6, performed by KAU of KSA and AUB, Lebanon, evaluated the protection in *Eimeria*-challenged broilers by single intervention, using continuous *ad libitum* administration in drinking water of the natural EOBWE, at a dilution of 0.025%. The challenge was esophageal, at different ages of 14, 21, 28, and 35 days, with equivalent number of sporulated oocytes of eight *Eimeria* spp. (*E. acervulina, E. brunetti, E. hagani, E. maxima, E. mivati, E. necatrix, E. praecox, and E. tenella*), totaling to $1.76x10^5$ oocysts/bird. The four treatments were: G1 (Unchallenged – Untreated with EOBWE), G2 (Unchallenged – Treated with EOBWE), G3 (Challenged – Untreated with EOBWE), and G4 (Challenged-Treated with EOBWE). Equal number of birds (n=10) were sacrificed at six days-post the time allocated for each challenge for assessment of broiler's production parameters, intestinal lesion scores and oocyte counts.

Experiment #7, performed at isolation unit of Poulpharm BVBA, Belgium, evaluated the protection in broilers, challenged by sporulated oocytes of six *Eimeria* spp. at single age of 17 days using Maxiban in feed versus continuous *ad libitum* administration in drinking water of the natural EOBWE, at a dilution of 0.025%. The esophageal challenge was with the following number of oocytes: *Eimeria acervulina* ($7.4x10^4$), *E. maxima* ($1.6x10^3$), *E. tenella* ($6.8x10^3$), *E. mitis* ($7.8x10^3$), *E. necatrix* ($7.0x10^3$), and *E. praecox* ($7.0x10^3$). The three treatments, each with eight replicate cages with five birds/cage, were: TRT #1 (Challenged - Untreated), TRT #2 (Challenged - Treated with Maxiban), and TRT #3 (Challenged - Treated with EOBWE). Birds were sacrificed at six days-post challenge for assessment of the production parameter, intestinal lesion score, and oocyte output.

Experiment #8, performed at isolation units of Nagpar Veterinary College, India, evaluated the protection in broilers, challenged by contaminating the floor of pens at stocking time of one-day old chicks, with equivalent number of local *Eimeria* spp., totaling to $4x10^5$ sporulated oocytes/m^2, and treated intermittently (3 d/wk) with different dilutions of EOBWE (0.005, 0.010, and 0.025 %) vs. treatment with Salinomycin in feed. The five treatments were: TRT #1 (Challenged-Untreated), TRT #2 (Challenged-Treated with 0.005% EOBWE), TRT #3 (Challenged-Treated with 0.010% EOBWE), TRT #4 (Challenged-Treated with 0.025% EOBWE), and TRT #5 (Challenged-Treated with salinomycin supplemented feed). Each treatment had duplicate pens, with 20 birds/pen. Six birds were sacrificed per pen at each of 28 and 35 days of age for assessment of production parameters, histopathological lesions in the intestine, and oocyte output/g of feces.

Experiment #9, performed by AUB and KAU, aimed at evaluation of dual approach, for protection of broilers against sporulated Oocytes - contaminated floor, by WSD vs. chlorine disinfection and intermittent treatment (3 d/week) with 0.025% EOBWE in drinking water vs. Maxiban in feed. The floor was contaminated with a total sporulated oocytes of $4x10^5$/m^2 of equivalent number of oocytes of eight *Eimeria* spp., followed by disinfection with invented WSD vs. chlorine disinfectant. The four treatments, with duplicate pens/treatment, and 500 birds/pen,

were: TRT #1 (WSD disinfected floor – EOBWE in drinking water), TRT #2 (WSD disinfected floor – Maxiban in feed), TRT #3 (chlorine disinfected floor – EOBWE), and TRT #4 (chlorine disinfected floor – Maxiban in feed). The oocyte output was determined at 14, 21, 28, and 35 days of age, and the FCR, body weight, cumulative mortality, and economical analysis were calculated by the 35th day of age.

Experiment #10, performed in the field by ANC Hayvan, Turkey, aimed at evaluation of the dual approach for controlling recurrent *E. maxima* outbreak on Turkish farms. Two treatments were compared: TRT #1 (WSD decontamination of farm – intermittent administration of 0.0125% EOBWE in water, 3 d/wk) vs. TRT #2 (Classical disinfectant – administration of salinomycin in feed). The size of broiler flocks in Treatments 1 and 2 were 10,400 and 23,200 birds, respectively.

Experiment #11, performed at Garo's farm, Zahle, Lebanon, aimed at evaluation of dual approach for controlling coccidiosis. The two treatments, with two barns/treatment, and between 7,000 to 13,000 birds/barn, were: TRT #1 (WSD for surface decontamination – intermittent administration in water of 0.025% EOBWE, 4d/wk) vs. TRT #2 (Omnicide disinfectant – Yumamycin in feed and Coccisol in water).

Experiment #12, performed at Adel's Farm, Baalbek, Lebanon, evaluated the dual approach. The two treatments, with one barn/treatment, and 15,600 birds/barn, were: TRT #1 (WSD for surface decontamination – intermittent administration of 0.025% EOBWE in water, 4 d/wk) vs. TRT #2 (formaldehyde disinfectant – administration of Monensin in grower feed and salinomycin in finisher feed).

Experiment #13, performed by Dremax kft, Hungary, compared two treatments on broiler farm with long history of *E. acervulina* challenge, including three barns/treatment, and 17,000 birds/barn. The two treatments were: TRT #1 (WSD decontamination - Concurrent administration of Maxiban in feed and intermittent 0.025% EOBWE in drinking water, 3 d/wk), and TRT #2 (WSD – only Maxiban in feed).

RESULTS

Experiment #1 resulted in a positive correlation between contact time of invented WSD (3% or 4%) and % lysis of sporulated oocytes of *E. tenella*.

Experiment #2 showed that the contact of sporulated oocytes of *E. tenella* with invented WSD (3%) for a period of 120 minutes resulted in 99% loss of infectivity in day-old chicks.

Experiment #3 proved that a five min contact time of WSD (3%) with tested bacteria and viruses, in presence of interfering organic matter (1 % skim milk) resulted in 100% inhibition, except of the 96.6% inhibition against *Streptococcus* organism.

Experiment #4 showed that the higher the concentration of invented EOBWE the shorter was the needed contact time to lyse the sporulated oocytes of eight *Eimeria* spp.

Experiment #5 proved that the EOBWE inhibitory effect on tested viruses (AI and NDV) was higher than that on tested bacteria, and that the EOBWE - inhibitory effect on tested viruses was not affected by the interference with 1% organic matter, while the bacterial inhibition was reduced by the organic matter.

Experiment #6 showed that the continuous treatment by the invented EOBWE in drinking water of broilers that are challenged esophageally at different ages (14, 21, 28, and 35 days), by sporulated oocytes of eight *Eimeria* spp. compared to untreated-challenged resulted in significant reduction in intestinal lesion scores, reduction in intestinal oocyte counts, and improvement in production parameters of Wt., FCR, and mortality.

Experiment #7 proved that the continuous treatment by either the invented EOBWE in drinking water or by Maxiban in feed to broilers challenged esophageally at same age (16 days), by sporulated oocytes of six *Eimeria* spp. compared to untreated-challenged resulted in lower oocyte output, and similar improvement in growth compared to untreated birds.

Experiment #8 demonstrated that the intermittent treatment by EOBWE, at higher concentrations of 0.0125% or 0.0250%, or the treatment by salinomycin, to broilers raised on floor contaminated by sporulated oocytes of local Indian *Eimeria* strains resulted in similar significant improvements compared to controls that were similarly challenged but deprived of medication. The significant improvements ($P<0.05$) were in growth, FCR, and reduced intestinal lesions and oocyte output.

Experiment #9 proved that the dual approach by WSD/intermittent EOBWE in drinking water or WSD/Maxiban in feed resulted in similar oocyte output at 14, 21, and 28 days of age in broilers that were raised on floor contaminated by sporulated oocytes of eight *Eimeria* spp. The dual approach of WSD/EOBWE treatment resulted in better improvement in production parameters compared to chlorine/intermittent EOBWE and chlorine/Maxiban treatments.

Experiment #10 proved that the dual approach treatment with invented WSD/intermittent EOBWE on broiler farm, with history of *E. maxima* challenge, resulted in 10% higher performance than dual treatment with commercial disinfectant/Salinomycin in feed.

Experiment #11 showed that the dual approach treatment of broilers with invented WSD/intermittent EOBWE, 4 d/wk vs. Omnicide/Yumamycin + coccisol resulted in the following percent difference in improvement of production by the invented approach: FCR 21.9% lower, mortality 15.3% lower, and live body wt. 12.3% higher.

Experiment #12 showed that the treatment of broilers with invented WSD/intermittent EOBWE, 4 d/wk versus formaldehyde/Monensin in grower feed plus Salinomycin in finisher feed resulted in the following % difference in improvement of production and reduction in oocyte output by the invented approach: FCR 2.1% lower, mortality 10.1% lower, live body wt. 2.5% higher, and oocyte output 8.7% lower.

Experiment #13 The European Efficiency Factor (EEF) was improved by 8% in flocks subjected to Treatment I (WSD – Concurrent supplementation of Maxiban in Feed and intermittent EOBWE in drinking water, 3 d/wk) compared to birds in Treatment II (WSD – Only Maxiban in feed). The typical lesions for *E. acervulina*, a recurrent infection on this farm, were absent in autopsied birds of Treatment I but not in birds of Treatment II.

CONCLUSION

The compiled data of this global research led to comprehensive control of poultry coccidiosis, manifested in significant reduction of oocyte output and its associated *Eimeria* lesions, and consistent improvement of the chicken performance.

REFERENCES

1. Deutsche Veterinärmedizinische Gesellschaft (DVG), Gießen. Richtlinien für die Prüfung von Desinfektionsverfahren und chemischen Desinfektionsmitteln (Guidelines for testing disinfection procedures and chemical disinfectants) Verlag der, 2007.

2. Kassaify, Z., Gerges, D.D., Jaber, L.S., Hamadeh, S.K. Saliba, N.A., Talhouk S.N. and Barbour, E.K. Bioactivity of *Origanium syriacum* essential oil against *Candida albicans*. J. Herbs Spices Med. Plants. 14: 185-199. 2008.

3. Krull, W., and Barbour, E.K. Method for Controlling Poultry Coccidiosis, US Patent no. 9,610,314 B1. 2016.

4. McDougald, L.R., and Seibert, B.P. Residual activity of anticoccidial drugs in chickens after withdrawal of medicated feeds. Vet. Parasitol., 74: 91-99. 1998.

5. Muthamilsevan, T., Kuo, T-F., Wu, Y.C., and Yang, W.C. Herbal remedies for coccidiosis control: A review of plants, compounds, and anticoccidial actions. Evid. Based Compliment. Alternat. Med., 2016: 1-19. 2016.

6. Ruff, M.D., and Danforth, H.D. Resistance of coccidian to medications. Proc. XX World's Poult. Congr.., II, 427-430. 1996.

7. Williams R.B. (1999). A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. Int. J. Parasitol., 29:1209-1229. 1999.

DUAL APPROACH HYPOTHESIS FOR CONTROL OF COCCIDIOSIS IN POULTRY: SEQUENCE OF SIGNIFICANCE TESTING

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ABSTRACT

The purpose of the sequence of significance testing is to determine if the results of statistical analysis of data, compiled from 13 experiments, are in favor of a dual approach hypothesis for control of coccidiosis. The dual approach hypothesis is based on the belief that an application of an invented wide spectrum disinfectant (WSD) to inactivate the oocysts of *Eimeria* spp. and other economic pathogens on poultry farm surfaces, and another intermittent application in the drinking water of an invented emulsion of natural essential oil blend in water extract (EOBWE) of plants can reach to a comprehensive control of coccidiosis and sustainability of production. *In vitro* experiments on lysis of oocysts by different concentrations of the invented WSD and EOBWE proved their significant lytic effects. Another experiments accomplished in isolation facilities, related to protection by the dual approach, against controlled challenges by the sporulated oocysts of multiple *Eimeria* spp., esophageal or through contaminated floor of barns, resulted in consistent trend of statistical significance of protection compared to controls, deprived of the dual approach. Statistical analysis of data resulting from field applications of the dual approach for control of coccidiosis, on farms with recurrent *Eimeria* challenges, were in support of the set hypothesis. The frequency of significance of data, resulting from the 13 experiments, and that are in favor of the dual approach hypothesis, will be presented discussed during the poster session.

INTRODUCTION

Most inventions of products that provide a solution to a poultry health problem around the world are initiated by imagination that led to hypotheses (5). A sound verification of the invention, for application in a disease control program of poultry, is strengthened by extensive compilation of scientific research from different laboratories, isolation facilities, and field trials performed under diversified environments of different countries, followed by subjecting its data to sequence of statistical testing for determining the level of significance of the obtained results (4). The consistency in reproducibility of results, based on the sequence of significance testing of the data extracted from the compiled researches, will determine either the acceptance or the rejection of the hypothesis (1).

This work aimed at verification of a dual approach hypothesis, based on the belief that 'First, an application of an invented WSD to inactivate the oocysts of *Eimeria* spp. and other economic pathogens on poultry farm surfaces, and second, an intermittent administration in the drinking water of an EOBWE of plants can reach to a comprehensive control of coccidiosis and sustainability of production. The verification of this dual hypothesis will be solely relying on the sequence of statistical significance testing of the data, generated from the 13 experiments that were collectively performed in nine countries.

MATERIALS AND METHODS

The materials and methods of the compiled 13 experiments aimed at verification of dual approach hypothesis that is based on intervention with an invented WSD, to decontaminate the surfaces of rearing area, followed by intermittent supplementation of drinking water with an invented EOBWE. The invented WSD is a mixture of phenol derivatives, organic solvents, organic acids, inorganic acids and an anionic surfactant, while the invented EOBWE is a

mixture of essential oils, emulsified in water extracts of herbs (3).

Experiment #1, performed at Leipzig University, Germany, studied the % lysis of E. tenella sporulated oocytes (25,000 oocytes/mL) by contact with 3% and 4% dilutions of invented WSD for periods of 30 to 120 minutes vs. control oocytes deprived of contact with WSD (2).

Experiment #2, performed also at Leipzig University, Germany, studied the infectivity in day-old chicks (N = 10/treatment) by sporulated oocytes of E. tenella (2000 oocytes/chick) that were previously contacted with 3 % WSD for 120 min (Treatment #1) vs. infectivity by non - contacted Oocytes with WSD (Treatment #2), following the guidelines of the German Veterinary Medicine Society, 2007 (2), while controls were deprived of any challenge (Treatment #3).

Experiment #3, performed by Opticon CO.-Switzerland and American University of Beirut- AUB, examined the susceptibility of poultry - associated bacteria and viruses upon contact with 3% dilution of invented WSD. The tested bacteria were E. coli, Streptococcus spp., Salmonella Enteritidis, and Proteus mirabilis, while the tested viruses were Newcastle Disease virus (lentogenic) and avian influenza (H9N2). The bacterial count before contact was adjusted to 2.0x107 CFU/ mL, while the viral HA units before contact was set at HA units of 4.0. Each test was performed in triplicate.

Experiment #4, performed at AUB, determined the needed contact time between Oocytes and different dilutions of EOBWE for obtaining a maximum lysis. The total oocyst number in each test was 25,000 /mL of equivalent number of eight *Eimeria* spp. (*E. acervulina*, *E. brunetti*, *E. hagani*, *E. maxima*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella*). The developed EOBWE was used in four different dilutions of 0.005, 0.010, 0.020, and 0.100 %. The contact times were 30, 60, 90, 120, and 180 min. Control oocytes count of 2500/mL were kept in contact with saline. All tests were accomplished in duplicate.

Experiment #5, performed by Opticon Co., Switzerland and AUB, aimed at evaluation of the susceptibility of poultry-associated bacteria and viruses to invented EOBWE, using the same above protocol, presented under Experiment #3, except that each test was accomplished in triplicate.

Experiment #6, performed by KAU of KSA and AUB, Lebanon, evaluated the protection in *Eimeria* - challenged broilers by single intervention, using continuous ad libitum administration in drinking water of the natural EOBWE, at a dilution of 0.025%. The challenge was esophageal, at different ages of 14, 21, 28, and 35 days, with equivalent number of sporulated oocytes of eight *Eimeria* spp. (*E. acervulina*, *E. brunetti*, *E. hagani*, *E. maxima*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella*), totaling to 1.76x105 oocysts/bird. The four treatments were: G1 (Unchallenged – Untreated with EOBWE), G2 (Unchallenged – Treated with EOBWE), G3 (Challenged –Untreated with EOBWE), and G4 (Challenged-Treated with EOBWE). Equal number of birds (n=10) were sacrificed at six days-post the time allocated for each challenge for assessment of broiler's production parameters, intestinal lesion scores and oocyte counts.

Experiment #7, performed at isolation unit of Poulpharm BVBA, Belgium, evaluated the protection in broilers, challenged by sporulated oocytes of six *Eimeria* spp. at single age of 17 days, using Maxiban in feed versus continuous *ad libitum* administration in drinking water of the natural EOBWE, at a dilution of 0.025%. The esophageal challenge was with the following number of oocytes: *E. acervulina* (7.4x104), *E. maxima* (1.6x103), *E. tenella* (6.8x103), *E. mitis* (7.8x103), *E. necatrix* (7.0x103), and *E. praecox* (7.0x103). The three treatments, each with eight replicate cages with five birds/cage, were: TRT #1 (Challenged-Untreated), TRT #2 (Challenged-Treated with Maxiban), and TRT #3 (Challenged-Treated with EOBWE). Birds were sacrificed at six days-post challenge for assessment of the production parameter, intestinal lesion score, and oocyte output.

Experiment #8, performed at isolation units of Nagpar Veterinary College, India, evaluated the protection in broilers, challenged by contaminating the floor of pens at stocking time of one-day old chicks, with equivalent number of local *Eimeria* spp., totaling to 4x105 sporulated oocytes/m^2, and treated intermittently (3 days/week) with different dilutions of EOBWE (0.005, 0.010, and 0.025 %) vs. treatment with Salinomycin in feed. The five treatments were: TRT #1 (Challenged-Untreated), TRT #2 (Challenged-Treated with 0.005% EOBWE), TRT #3 (Challenged-Treated with 0.010% EOBWE), TRT #4 (Challenged-Treated with 0.025% EOBWE), and TRT #5 (Challenged-Treated with salinomycin supplemented feed). Each treatment had duplicate pens, with 20 birds/pen. Six birds were sacrificed per pen at each of 28 and 35 d of age for assessment of production parameters, histopathological lesions in the intestine, and oocyte output/g of feces.

Experiment #9, performed by AUB and KAU, aimed at evaluation of dual approach, for protection of broilers against sporulated oocytes - contaminated floor, by WSD vs. chlorine disinfection and intermittent treatment (3 days/week) with 0.025% EOBWE in drinking water vs. Maxiban in feed. The floor was contaminated with a total sporulated oocytes of 4x105/m2 of equivalent number of oocytes of eight

Eimeria spp., followed by disinfection with invented WSD vs. chlorine disinfectant. The four treatments, with duplicate pens/treatment, and 500 birds/pen, were: TRT #1 (WSD disinfected floor – EOBWE in drinking water), TRT #2 (WSD disinfected floor – Maxiban in feed), TRT #3 (chlorine disinfected floor – EOBWE in drinking water), and TRT #4 (chlorine disinfected floor – Maxiban in feed). The oocyte output was determined at 14, 21, 28, and 35 days age, and the FCR, body weight, cumulative mortality, and economical analysis were calculated by the 35th d of age.

Experiment #10, performed in the field by ANC Hayvan, Turkey, aimed at evaluation of the dual approach for controlling recurrent E. maxima outbreak on Turkish farms. Two treatments were compared: TRT #1 (WSD decontamination of farm – intermittent administration of 0.0125% EOBWE in drinking water, 3 days/week) vs. TRT #2 (Classical disinfectant – administration of salinomycin in feed). The size of broiler flocks in Treatments 1 and 2 were 10,400 and 23,200 birds, respectively.

Experiment #11, performed at Garo's farm, Zahle, Lebanon, aimed at evaluation of dual approach for controlling coccidiosis. The two Treatments, with two barns/Treatment, and between 7,000 to 13,000 birds/barn, were: TRT #1 (WSD for surface decontamination – intermittent administration of 0.025% EOBWE in drinking water, 4 days/week) vs. TRT #2 (Omnicide disinfectant – Yumamycin in feed and Coccisol in water).

Experiment #12, performed at Adel's Farm, Baalbek, Lebanon, evaluated the dual approach. The two Treatments, with one barn/treatment, and 15,600 birds/barn, were: TRT #1 (WSD for surface decontamination – intermittent administration of 0.025% EOBWE in drinking water, 4 days/week) vs. TRT #2 (formaldehyde disinfectant – administration of Monensin in grower feed and salinomycin in finisher feed).

Experiment #13, performed by Dremax kft, Hungary, compared two treatments on broiler farm with long history of *E. acervulina* challenge, including three barns/Treatment, and 17,000 birds/barn. The two treatments were: TRT #1 (WSD decontamination - Concurrent administration of Maxiban in feed and intermittent 0.025% EOBWE in drinking water, 3d/wk), and TRT #2 (WSD – only Maxiban in feed).

STATISTICAL METHODS

All 13 experiments followed the Completely Randomized Design (CRD). The quantified frequency-means were compared statistically by Chi-Square, while the other non-frequency means were compared by ANOVA, followed by conservative Tukey's test. The level of significance was set at $P<0.05$.

RESULTS

The sequence of significance, based on statistical analyses of 20 quantified variables in the compiled 13 experiments, are:

Experiment #1. *E. tenella*-oocytes lysis by WSD vs. Control oocytes lysis ($P<0.05$).

Experiment #2. Infectivity in day old chicks of *E. tenella*-oocytes contacted with WSD vs. non-contacted oocytes ($P<0.05$).

Experiment #3. Bacterial and viral inhibition by contact with WSD for 5 min vs. non contacted organisms ($P<0.05$).

Experiment #4. Higher concentration of EOBWE results in shorter time for lysis of oocytes of eight *Eimeria* spp. ($P<0.05$).

Experiment #5. Contact with EOBWE, in presence of 1% organic matter for a period of 15 minutes, results in 100 % inhibition of NDV and H9N2 and lower range of inhibition in bacteria, that were both significantly different than result of control organisms deprived of contact with EOBWE ($P<0.05$).

Experiment #6. EOBWE resulted in better respective growth of unchallenged and challenged broilers with eight *Eimeria* spp. compared to unchallenged and challenged birds deprived of EOBWE ($P<0.05$). EOBWE reduced significantly the intestinal lesion score and oocytes, FCR, and mortality of challenged birds compared to similarly challenged birds deprived of EOBWE ($P<0.05$).

Experiment #7. The intestinal lesion scores due to *E. acervulina* and *E. maxima* were not reduced significantly by EOBWE or Maxiban compared to similarly challenged birds deprived of coccidiostats ($P>0.05$). Both Maxiban and EOBWE reduced the output of *E. maxima* compared to similarly challenged birds deprived of coccidiostat ($P<0.05$). EOBWE reduced insignificantly the *E. acervulina* output compared to similarly challenged birds deprived of coccidiostat ($P>0.05$). Higher weight resulted from EOBWE or Maxiban treatment of challenged birds compared to similarly challenged birds deprived of coccidiostat ($P<0.05$).

Experiment #8. Oocyte output, histopathologic lesions, and FCR were all reduced significantly by either EOBWE (0.005- 0.025%) or by Salinomycin compared to similarly challenged broilers deprived of coccidiostats ($P<0.05$). Weight was increased significantly by EOBWE (0.0125 or 0.025%) and by salinomycin compared to similarly challenged broilers deprived of coccidiostat ($P<0.05$).

Experiment #9. Dual approach by WSD/EOBWE or WSD/Maxiban resulted in

significantly lower oocyte output compared to dual approach by chlorine/EOBWE or chlorine/Maxiban in birds reared on oocyte-contaminated floor ($P<0.05$). Dual approach by WSD/EOBWE resulted in lowest FCR and highest body weight compared to other three treatments ($P<0.05$).

Experiment #10. Dual approach by WSD/EOBWE on farm with history of *E. maxima* resulted in 10% better growth performance than dual approach by classical disinfectant/salinomycin (inapplicable data for statistical comparison, one house/treatment).

Experiment #11. Dual approach by WSD/EOBWE compared to Omnicide/Yumamycin + Coccisol on the farm resulted in lower mortality ($P<0.05$), lower FCR ($P<0.05$), and higher body weight ($P<0.05$).

Experiment #12. Dual approach by WSD/EOBWE compared to formaldehyde/Monensin on the farm resulted in significantly lower oocyte output ($P<0.05$). Dual approach by WSD/EOBWE compared to formaldehyde/Monensin on the farm resulted in insignificant improvement of weight, FCR, and mortality ($P>0.05$).

Experiment #13. Dual approach by WSD/EOBWE + Maxiban compared to WSD/Maxiban alone on farm with history of *E. acervulina* outbreak resulted in 8% improvement in EEF ($P<0.05$).

CONCLUSION

The frequency in the above sequence of significance testing ($P<0.05$) of data was in favor of the hypothesis, in which 18 out of 21 comparison of means were significantly different at $P<0.05$ (85.7%). It is worth noting that analyzed 21 means accumulated from the 13 experiments and contributing favorably to the set hypothesis, are heterogenous in their weight of support to the hypothesis.

REFERENCES

1. Casadevall, A. Reproducible Science. Infect Immun. 78(12): 4972–4975. 2010.

2. Deutsche Veterinärmedizinische Gesellschaft (DVG), Gießen. Richtlinien für die Prüfung von Desinfektionsverfahren und chemischen Desinfektionsmitteln (Guidelines for testing disinfection procedures and chemical disinfectants) Verlag der. 2007.

3. Krull, W., and Barbour, E.K. Method for Controlling Poultry Coccidiosis, US Patent no. 9,610,314 B1. 2016.

4. Marcus, E. Credibility and Reproducibility Cell, 159 (5): 965–966. 2014.

5. Park, K. Drug Delivery Research: The Invention Cycle. Mol Pharm. 13(7): 2143–2147. 2016.

SALMONELLA VACCINATION OF BROILERS FROM PEN TRIALS TO FIELD DATA

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SUMMARY

Salmonella foodborne illness continues to be a significant public health concern. The target set by United States public health agencies is 11.4 cases/100,000 people by 2020, while the actual rate in 2014 was 15.45 cases. Vaccination of broiler chickens with live attenuated *Salmonella* vaccines has been successfully demonstrated in laboratory challenge studies.

A floor pen study (24 pens) with 1,200 commercial broilers (50 birds/pen) were vaccinated twice with a commercially available live *Salmonella typhimurium* (MeganVac 1®) vaccine at one day (spray) and 14 days (drinking water). Four days post vaccination 50% were marked with identifying tags and orally gavaged with a nalidixic acid resistant *S. heidelberg* (S.H.) (10^5 cfu/chick). At 42 days ten horizontal challenged (not tagged) birds were selected from each pen, humanly euthanized, and ceca aseptically collected. Ceca were cultured for S.H. prevalence and enumeration by the MPN method (1). The live S.T. vaccine lowered both S.H. prevalence (16% control vs. 0% vaccinated) and significantly lowered the number of S.H. (0.15 cfu/g control vs. 0.0 cfu/g vaccine) in each of the positive ceca.

A U.S. broiler company with a *Salmonella* Group D arising in broiler farms chose to test the vaccine after reviewing the floor pen results. Prior to vaccination, three randomly selected broiler farms per week for four weeks were sampled by collecting five ceca/farm at 35 days of age. Vaccination began at one day (spray) and 14 days (drinking water). Five ceca were collected at 35 days of age and cultured in same manner as pen trial ceca without nalidixic acid in the XLT-4.

The live vaccine did not lower overall *Salmonella* prevalence (39% pre vaccination vs. 28% post vaccination). However, the vaccine significantly reduced Group D *Salmonella* prevalence (17% pre vaccination vs. 3% post). Also, positive ceca from vaccinated broilers had significantly less Group D *Salmonella* (15.7 CFU-MPN/g pre-vaccination vs. 1.0 CFU-MPN/g post vaccination).

Study results demonstrated first by floor pen data and confirmed by field data, that live *Salmonella* vaccine, given twice, can be a valuable tool in reducing potential risks of *Salmonella* serovars often involved in human foodborne illness originating in broiler chickens.

INTRODUCTION

Salmonellosis remains a major cause of human foodborne illness, and continues to be a significant health concern. The Centers for Disease Control and Prevention has estimated that nontyphoidal *Salmonella* species are second only to norovirus as a leading cause of foodborne illness in the United States, causing approximately 11% of all domestically-acquired foodborne illnesses, and that *Salmonella* species are the leading cause of hospitalizations (35%) and deaths (28%) from foodborne illnesses (4). The main obstacle to *Salmonella* control in the poultry industry is the abundance of the bacteria. Once *Salmonella* gets onto a farm, they spread rapidly due to infected chickens and rodents serving as carriers. *Salmonella* carriers constantly shed the bacteria and contaminate the environment (3). While many different measures have been recommended in the poultry industry, vaccination with live-attenuated and inactivated vaccines in parent breeders is likely to have a central role in the reduction of *Salmonella* in commercial operations by increasing the passive immunity of birds and blocking the horizontal transmission of *Salmonella*.

In broilers, the advantage of live-attenuated vaccines is that attenuated *Salmonella* bacteria replicate, colonize and invade intestinal and visceral organs of inoculated chickens, producing long-lasting protective immunity (2). Field evidence suggests that vaccination with a live *Salmonella* Typhimurium (S.T.) vaccine can cross protect against other serovars and reduce loads and/or prevalence. A significant level of protection has been observed against challenge with *Salmonella* strains of group D in chickens vaccinated with live S.T. vaccine, such as MeganVac, when compared with nonvaccinated chickens. This cross protection is possibly caused by the expanded memory responses and the increasing level of immune response to *Salmonella* common immunogens may be induced

by the boosting of the vaccine at 10 to 14 days, leading to strong cross-protection against other serogroups of *Salmonella* (3).

These studies were done to demonstrate cross protection of the S.T. vaccine for S.H. challenge in a controlled pen study, then to apply this to a large broiler integration in Southeast United States.

MATERIALS AND METHODS

Experimental design. Pen Study- In this 24 pen study, 1,200 Ross x Ross male broilers were assigned to two treatment groups, with 12 replicate blocks per treatment. Birds received routine vaccinations (HVTSB1) at hatchery, and were vaccinated with an approved broiler coccidiosis vaccine, and MeganVac 1 (Elanco Animal Health, Greenfield, IN) by coarse spray (0.25mL/bird) on day one and boosted in the drinking water (recommended 1 dose) on day 10 at SPRG. Fifty broiler chicks were allocated into each floor pen measuring 5x10 (1.00 ft^2/bird). Water and feed were provided *ad libitum*. Field Study- A broiler company in southeastern United States vaccinated all broilers with MeganVac 1 by coarse spray (0.25mL/bird) on day one, and boosted the vaccine at recommended dose in the drinking water on day 10. Five ceca were collected from each house at approximately five weeks of age.

***Salmonella* challenge.** Pen Study- On day 4, 25 seeder chicks per pen were tagged, color-coded (for identification), and orally dosed by gavage with a 10^5 CFU/chick nalidixic acid resistant S.H. Field Study- Birds in field study were challenged naturally from *Salmonella* in the environment.

Sample collection. Pen Study- On day four liver/spleen (pooled) and cecal samples were collected from five birds from both treatment groups, and submitted for vaccine isolation and identification. On day 42 and 43, cecal samples were collected from 10 horizontal-exposed (non-tagged) birds per pen, and one bootsock was collected per pen. Field Study- At approximately five weeks of age five ceca were collected per house. A total of 54 cecal samples were collected pre-vaccination from nine different farms. Post-vaccination a total of 408 cecal samples were collected from 48 farms, including the nine farms sample pre-vaccination.

Laboratory/Sample processing. Liver/spleen (pooled) and cecal samples submitted for vaccine isolation and identification were stomached and tetrathionate brilliant green broth was added to each sample. The samples were incubated overnight at 42°C. A 10 uL loopful was struck from the incubated samples to XLT-4 agar and incubated overnight at 37°C. MeganVac isolates were identified as small colorless colonies (H_2S negative). At pen study and field study termination, cecal samples were collected, weighed, stomached and tetrathionate brilliant green broth was added. Three 1mL aliquots were removed from cecal samples for MPN analysis. Bootsocks were stomached in 100 mL of tetrathionate brilliant green broth. All samples (bootsocks and ceca) were incubated overnight at 42°C. A 10 uL loopful was struck from the incubated samples to XLT-4 + nalidixic acid agar and incubated overnight at 37°C. Up to three black colonies were selected and confirmed as S.H. using poly-O *Salmonella* specific antiserum. For all cecal samples, a 1mL sample of stomachered tetrathionate brilliant green broth was transferred to three adjacent wells in the first row of a 96 well 2 mL deep block. A 0.1 mL aliquot of sample was transferred to 0.9 mL of tetrathionate broth in the second row, and the process was repeated for the remaining rows (to produce 5 ten-fold dilutions), and the blocks incubated (24 hours at 42°C). A 1 uL sample from each well of the blocks was transferred onto XLT-4 agar containing nalidixic acid with a multi-channel pipettor, incubated plates (37°C for 24 hours), recorded final dilution of each sample, and entered in MPN calculator (1). Suspect *Salmonella* isolates were confirmed by poly-O *Salmonella* specific antiserum. Lab methods and sample processing were the same in both the pen study and the field study, only the field study was cultured without nalidixic acid in the XLT-4.

Statistical analysis. *Salmonella* prevalences were compared using Fisher's exact test. Ceca *Salmonella* prevalences and MPNs were compared using generalized estimating equations (GEE) logistic and linear models, respectively, to account for the correlation between responses of birds from the same pen. MPN values were log-transformed prior to statistical analysis. Post-hoc pairwise comparisons between treatments were performed using the Bonferroni procedure to limit the type I error rate to 5% over all comparisons. All statistical testing assumed a two-sided alternative hypotheses, and a $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Pen study. The MeganVac 1 did colonize internal organs by four days of age demonstrated by 80% of liver/spleen samples collected being positive for the vaccine strain. This study was designed to give a very mild challenge by administering S.H. to broilers at four days of age. The challenge was effective, as bootsocks in 100% of challenge control pens were SH positive by 14 days of age. There was a statistically significant reduction in the number of bootsock environmental positives at 14 days in pens of MeganVac 1 vaccinated broilers (50%). Although not

statistically significant, by 42 days of age 50% of the pens vaccinated with MeganVac 1 were still negative by environmental bootsocks. Ceca prevalence also demonstrated this was a mild to moderate challenge since only 16% of horizontal exposed birds in the challenge control pens were positive at 42 days of age (Table 1). The horizontal exposed birds in the MeganVac 1 vaccinated pens had no positive ceca at 42 days showing a significant reduction when compared to the challenge control pens. There was also a significant reduction in the level S.H. per gram of ceca (Figure 1).

Field study. There was no significant difference in overall *Salmonella* prevalence between pre vaccination (38.9%) and post vaccination (28.4%). However, there was a significant reduction in the prevalence of Group D *Salmonella* isolation post vaccination (Table 2). The majority of the *Salmonella* isolated (22% for both pre and post vaccination) were group C2/C3, most likely *Salmonella* Kentucky. When the load of *Salmonella* per positive ceca were examined, there was no significant difference between pre and post vaccination. However, there was a significant reduction in MeganVac 1 vaccinated broilers in serogroup D *Salmonella*, 1.0 geometric mean MPN/g vs. 15.7 geometric mean MPN/g in the pre-vaccination ceca.

In summary, the goal of *Salmonella* control for broiler companies is to lower the level of *Salmonella* entering the processing plant. Therefore, enumeration of *Salmonella* level in ceca usually provides an indication of an intervention's effectiveness to lower the incoming load. In both the pen study and the field study MeganVac 1 showed a reduction in *Salmonella*, and confirmed cross protection against serogroup D *Salmonella* (3). Study results demonstrated first by floor pen data and confirmed by field data, that live *Salmonella* vaccine, given twice, can be a valuable tool in reducing potential risks of *Salmonella* human foodborne illness originating in broiler chickens.

REFERENCES

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

2. Dórea F. C., D. J. Cole, C. L. Hofacre, K. Zamperini, D. Mahtis, M. P. Doyle, M. D. Lee and J. J. Maurer. Effect of *Salmonella* vaccination of breeder chickens on contamination of broiler chicken carcasses in integrated poultry operations. Appl. & Environ. Micro. 79:7820-7825. 2010.

3. Hassan, J. O. and R. Curtiss. Development and evaluation of an experimental vaccination program using a live avirulent *Salmonella typhimurium* strain to protect immunized chickens against challenge with homologous and heterologous *Salmonella* serotypes. Infect. & Immun. 62:5519-5527. 1994.

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

Figure 1. Dot plots of *Salmonella* MPNs in positive ceca.

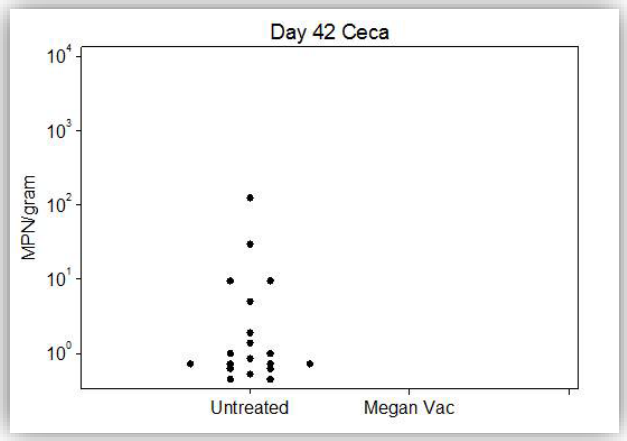

Table 1. *Salmonella* prevalences in ceca from 10 horizontal exposed birds/pen.

Treatment	Bootsocks (42 Days)*P	Bootsocks No. Positive (%)	Ceca (42 Days)	Ceca No. Positive (%)
No Treatment	12	11/12(91.7)*	120	19/120(15.8)[a]
Megan Vac 1	12	6/12(50.0)*	120	0/120(0)[b]

*Generalized estimating equations logistic model adjusted for clustering by pen. Percentages in columns with superscript in common do not differ with level of significance of 5%.

Table 2. *Salmonella* prevalences by serogroup for ceca samples collected from broiler chickens before and after implementation of the vaccination program.

Table 2. *Salmonella* Prevalences by Serogroup for Ceca Samples Collected from Broiler Chickens Before and After Implementation of a Vaccination Program

		Number Positive (%) by Serogroup				
	No. Samples	B	C2/C3	D	E	Total
Pre-vaccination	54	0(0.0)[a]	12(22.2)[a]	9(16.7)[a]	0(0)[a]	21(38.9)[a]
Post-vaccination	408	2(0.5)[a]	93(22.8)[a]	13(3.2)[b]	8(2.0)[a]	116(28.4)[a]

Within columns, percentages with a superscript in common do not differ with a level of significance of 5%

DIAGNOSIS OF AN ATYPICAL NEOPLASM IN A BACKYARD CHICKEN WITH RETROSPECTIVE ANALYSIS OF NEOPLASIA DIAGNOSED IN BACKYARD FLOCK SUBMISSIONS TO CALIFORNIA ANIMAL HEALTH AND FOOD SAFETY LABORATORY SYSTEM FROM 2008 – 2017

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SUMMARY

On September 28, 2017, a dead three-year-old backyard Silkie chicken was submitted to California Animal Health and Food Safety Laboratory System (CAHFS)-Turlock branch for necropsy, with a history of unknown skin lesions involving the entire body and severe weight loss. At necropsy, raised necrotic lesions involving the majority of the skin and multiple nodules in the liver, spleen, and bone marrow were noticed. Microscopically, stellate, spindle, and myxoid cells containing large lipid droplets were observed infiltrating the dermis and underlying a necrotic epidermis; with metastasis to liver, spleen, bone marrow, and ovary noted. PAS, Oil Red O, Ziehl-Neelsen, Congo red, Gram, and Von Kossa stains along with immunohistochemistry for pan cytokeratin, vimentin, S100, CD3, pp38, and Meq were used to classify the lesions. Positive vimentin IHC, along with large quantities of Oil Red O positive lipid droplets within the neoplastic cells were supportive of our diagnosis of liposarcoma. The incidence of neoplastic diseases diagnosed in backyard flock submissions to CAHFS system wide from 2008-2017 was also reviewed.

INTRODUCTION

Mesenchymal neoplasms of the skin include lipomas, liposarcomas, myelolipomas, fibromas, fibrosarcomas, hemangiomas, hemangiosarcomas, and myxosarcomas (11); with lipid deposition associated with the first three tumor types, respectively (7). In galliforms, epithelial neoplasms are uncommon; with lymphoma, adenocarcinoma, and squamous cell carcinoma being the most common forms of neoplasia observed (3, 10). Liposarcomas are rare in all avian orders, with few documented cases observed in a goose (*Anser anser domesticus*), three chickens (*Gallus gallus*), a monk parakeet (*Myopsitta monachus*), a budgerigar (*Melopsittacus undulatus*), an African Grey parrot (*Psittacus erithacus*), a partridge (*Perdix perdix*), a cockatiel (*Nymphicus hollandicus*), and two pigeons (*Columba livia*) (1, 2, 5, 6, 8, 9, 12, 13, 16, 18).

CASE REPORT

On September 28, 2017; one dead, three-year-old Silkie hen was presented to CAHFS, Turlock branch, for necropsy. Clinical history included unknown skin lesions involving the entire body, apparent blindness, and severe emaciation. The chicken died on September 15, 2017, and was submitted to the CAHFS Turlock laboratory for diagnostic evaluation. The hen was in poor post-mortem condition and visibly emaciated. Significant findings included nodules, ranging from 1 – 5 cm in length; present on feathered and non-feathered skin. The liver, spleen, and bone marrow contained multiple tan circular foci.

Oropharyngeal swab was negative for avian influenza virus by quantitative (real-time) reverse transcriptase polymerase chain reaction (qRT-PCR) test (National Animal Health Laboratory Network protocol, CAHFS, Davis laboratory). Selected organs were cultured for aerobic bacteria using proper media (5% Sheep's blood and MacConkey's agar), revealing no growth from the liver and spleen, and colonies compatible with *Staphylococcus* spp. from the skin. Tissue impression smears of nodules from the bone marrow, liver, skin, and spleen were taken for Gram stain and Ziehl-Neelsen stain, revealing no acid-fast organisms and rare numbers of Gram-positive cocci from the bone marrow, rare numbers of Gram-positive rods from the liver, large numbers of Gram-positive rods and cocci from the skin, and rare numbers of Gram-negative rods from the spleen. Tissue sections were collected for histopathology and processed using hematoxylin and eosin staining. Selected tissue sections were also stained with Congo red, periodic Acid-Schiff (PAS), Oil Red O, Von Kossa, Gram stain, Alcian blue, and Ziehl-Neelsen. Immunohistochemistry for pan cytokeratin (PanCK),

vimentin, S100, CD3, pp38, and Meq were also performed on select tissues.

Significant histological findings included a multifocal, non-encapsulated, irregularly infiltrative neoplasm; located in the subcutis and dermis, and underlying an ulcerative, necrotic epidermis with serofibrinous crust formation. Stellate, spindle, and myxoid cells were loosely arranged in whorls, of which >90% stained strongly positive for vimentin. Myxoid cells stained positive for mucopolysaccharides and glycoproteins with Alcian blue stain. Expanding the cells were large round to oval empty vacuoles, which were positive for lipid by Oil Red O stain. No acid-fast organisms were discovered with Ziehl-Neelsen stain and IHC revealed scattered CD3 and Meq positive lymphocytes, comprising <5% of the cell population, with no pp38 staining observed. Similar neoplastic cell populations were observed in the spleen, liver, ovary, and bone marrow, while PAS and Ziehl-Neelsen revealed no fungal or acid-fast organisms.

CAHFS records from January 1, 2008 to October 28, 2017 were analyzed to determine the prevalence and type of neoplastic diseases that were diagnosed system wide (Davis, Turlock, Tulare, and San Bernardino CAHFS Laboratories) in backyard chicken submissions. From 2008 to 2017, CAHFS received between 357 and 1,347 backyard submissions each year. In each year from 2008-2017, neoplasia was diagnosed in 6.4% to 19.5% of submissions. Twelve percent of all backyard submissions received at CAHFS from Jan 1, 2008- Oct 28, 2017 were diagnosed with neoplasia, with the three most frequently diagnosed neoplasms being Marek's disease (40%), adenocarcinoma (29%), and carcinoma (8.6%). In 2008, 2013, and 2017, Marek's disease was diagnosed in 36% to 54% of neoplasia cases, with adenocarcinoma diagnosed in 21.5% to 26.5% of neoplasia cases.

DISCUSSION

Differential diagnoses for this neoplasm of mesenchymal origin, as demonstrated by the intensely positive vimentin IHC and negative panCK IHC, include various sarcomas (liposarcoma, fibrosarcoma, and myxosarcoma) with metastasis demonstrating the tumors malignant nature. Oil Red O positive lipid vacuoles present amongst the neoplastic mesenchymal cells is supportive of a liposarcoma (1, 2, 5, 6, 8, 9, 12, 13, 16, 18). Xanthomas are non-neoplastic nodules consisting of giant cells, macrophages containing lipid, cholesterol clefts, and fibrous tissue. Xanthomas are known to be locally invasive, and have been associated with other lesions, such as lipomas and areas of chronic inflammation (4, 17). Because of the histologic similarities, xanthomatosis as a primary cause, or more likely in addition to liposarcoma could not be ruled out in this case. The possibility of retrovirus induced neoplasia cannot be ruled out in this case, as virus isolation or PCR was not attempted on neoplastic tissue. Negative PAS and acid fast (Ziehl-Neelsen) stains of the skin, spleen, and liver assisted in ruling out fungal or mycobacterial causes of the changes observed.

Admixed within the mesenchymal neoplasm were occasional CD3 and Meq positive T-lymphocytes in the skin and spleen, however the lack of lesions in the central and peripheral nervous system, lack of pp38 IHC staining, and scattered Meq IHC staining supports a diagnosis of subclinical Marek's disease.

Analysis of CAHFS records system wide demonstrates a 2.4 times increase in the diagnosis of neoplasia in backyard chickens, with an average of 10.5% over the ten-year span. The 2.4 times increase in neoplasia diagnosis; with Marek's disease diagnosis increasing by 14% in the last several years; is likely multifactorial in nature, involving the spread of the contagious and resistant infectious disease (14, 15), rare vaccination of backyard chickens, and an increase in susceptible chickens and infectious particles shed into the environment. Further studies are needed to fully explore the cause of increased neoplasia diagnosis and Marek's disease in backyard flocks in California. The increase in backyard submissions is likely a result of several factors, of which an increase in numbers of backyard flocks and an increased awareness of the CAHFS system likely play a role.

In summary, we conclude that the histological evidence of cellular morphology, staining, and IHC is compatible with a diagnosis of metastatic liposarcoma with overlying xanthomatosis, and subclinical Marek's disease. Liposarcomas are rare in avian species, and have not been diagnosed at CAHFS system wide in the last ten years of backyard flock submissions. Further research is required to elucidate the possible causes of increased neoplasia and Marek's disease in backyard California flocks.

REFERENCES

1. Doria-Torra, G., J. Martinez, M. Domingo, B. Vidana, M. Isidoro-Ayza, M. I. Casanova, and E. Vidal. Liposarcoma in Animals: Literature Review and Case Report in a Domestic Pig (*sus scrofa*). J. of Vet. Diagn. Invest. 27:196-202. 2015.

2. Doster, A. R., J. L. Johnson, G. E. Duhamel, T. W. Bargar, and G. Nason. Liposarcoma in a Canada Goose (*Branta canadensis*). Avian Dis. 31:918-920. 1987.

3. Garner, M. M. Overview of Tumors: Section II: A Retrospective Study of Case Submissions to a Specialty Diagnostic Service. In: Clinical Avian Medicine, Vol. 2, G. J. Harrison and T. Lightfoot, ed. Spix Publishing, Palm Beach, Fl. pp. 566-571. 2006.

4. Girolamo, N. D., E. P. Lane, F. Reyers, and B. R. Gardner. Subcutaneous Xanthomatosis in a Great White Pelican (*Pelecanus onocrotalus*). J. of Zoo and Wildlife Med. 45:153-156. 2014.

5. Graham, J. E., J. A. Werner, L. J. Lowenstine, S. T. Wallack, and L. A. Tell. Periorbital Liposarcoma in an African Grey Parrot (*Psittacus erithacus*). J. of Avian Med. and Surgery. 17:147-153. 2003.

6. Keller, K. A., S. Wilson, C. Plummer, T. N. Tully Jr. Diagnostic Challenge. J. of Exotic Pet Med. 20:64-66. 2011.

7. Latimer, K. S. Oncology. In: Avian Medicine: Principles and Applications, 1st ed., B. W. Ritchie, G. J. Harrison, and L. R. Harrison, ed. Wingers Publishing, Inc., Florida. pp. 641-645. 1994.

8. Mauldin, E. A. and Peters-Kennedy, J. Integumentary System. In: Pathology of Domestic Animals, Vol 1, M. Maxie, ed. Elsevier, St. Louis, MO. pp. 703-736. 2016.

9. Mohiddin, S. M., and K. Ramakrishna. Liposarcoma in a Fowl. Avian Dis. 16:680-684. 1972.

10. Pass, D. A. The Pathology of the Avian Integument: A Review. Avian Pathol. 18:1-72. 1989.

11. Reavill, D. R., and Schmidt, R. E. Avian Surgical Pathology. In: Laboratory Medicine: Avian and Exotic Pets, 1st ed., A. M. Fudge, ed. Elsevier Health Sciences, London, UK. pp. 133- 146. 1999.

12. Reece, R. L. Observations on Naturally Occurring Neoplasms in Birds in the State of Victoria, Australia. Avian Pathol. 21:3-32. 1992.

13. Reece, R. L. Some Observations on Naturally Occurring Neoplasms of Domestic Fowls in the State of Victoria, Australia (1977-87). Avian Pathol. 25:407-447. 1996.

14. Schat, K. A., and V. Nair. Marek's Disease. In: Diseases of Poultry, 13th ed., J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez, and V. Nair, ed. John Wiley & Sons, Inc., Ames, Iowa. pp. 515-552. 2013.

15. Sheldon Tai, S.-H., C. Hearn, S. Umthong, O. Agafitei, H. H. Cheng, J. R. Dunn, and M. Niikura. Expression of Marek's Disease Virus Oncoprotein Meq during Infection in the Natural Host. Virol. 503:103-113. 2017.

16. Shimonohara, N., C. H. Holland, T.-L. Lin, and W. L. Wigle. Naturally Occurring Neoplasms in Pigeons in a Research Colony: A Retrospective Study. Avian Dis. 57:133-139. 2013.

17. Souza, M. J., N. S. Johnstone-McLean, D. Ward, and K. Newkirk. Conjunctival Xanthoma in a Blue and Gold Macaw (*Ara ararauna*). Vet. Ophthal. 12:53-55. 2009.

18. Tully, T. N. Jr., J. M. Morris, R. S. Veazey, J. L. Oliver III, and T. G. Snider III. Liposarcomas in a Monk Parakeet (*Myiopsitta monachus*). J. of the Ass. of Avian Vet. 8:120-124. 1994.

SURVEILLANCE FOR DISEASES IN SMALL POULTRY FLOCKS IN ONTARIO

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INTRODUCTION

In Ontario, within the past five years, there has been a 20% increase in the number of registered non-quota / non-commercial poultry flocks (herein referred to as "small flocks"), with over 16,000 registered in 2016 through the Chicken Farmers of Ontario (CFO)(1). Between 2015 and 2016 alone, there was a 7% increase with an average flock size of 65 birds (1). Small poultry flocks are often under-serviced by veterinarians for many reasons, including low recognition of the value of veterinary care, limited financial resources, and lack of local access to veterinarians with poultry experience and / or poultry medications (including vaccines). These small flocks may have poor biosecurity practices, increased risk of contact with potential pathogen reservoirs in wild birds, limited vaccination, and may be located in close proximity to commercial poultry operations (2-7). For these reasons, small flocks may be at higher risk of becoming potential reservoirs of poultry and zoonotic pathogens, possibly representing a threat for commercial poultry operations and public health. Currently, there is a scarcity of data available concerning the prevalence of common infectious and non-infectious pathogens in small flocks in Ontario. With a recent disease outbreak in commercial poultry flocks in British Columbia (8), and earlier outbreaks in Californi (9), Italy (10), and the Netherlands (11) being epidemiologically linked to non-commercial flocks, there is a growing need for a better assessment of baseline disease prevalence in Ontario small poultry flocks.

MATERIAL AND METHODS

Over a two year period (October 2015 - September 2017), the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) and the University of Guelph, in collaboration with the Animal Health Laboratory (AHL) and the Ontario Animal Health Network, conducted a surveillance project to determine the prevalence of infectious agents in non-quota and non-commercial poultry flocks in Ontario. To be enrolled in the project, the flocks must have been located in Ontario, and could include chickens, turkeys, waterfowl and gamebirds. Submissions to the AHL) were encouraged by offering a full postmortem at a highly reduced fee. As per AHL policy, submissions required a referring veterinarian to receive the results. Owner's signed consent, and completion of a standardized husbandry and biosecurity questionnaire were also requirements of the submission.

In addition to a full postmortem examination including any ancillary testing as determined by the pathologist, a pre-set array of tests for infectious agents was conducted. Pre-set microbiological tests for each submission included isolation of *Campylobacter* spp. and *Salmonella* spp., including *S.* Pullorum and *S.* Gallinarum, from pooled ceca of each submission (all species), as well as quantitative PCR for *Brachyspira* spp. (pooled ceca, all species), avian bornavirus (pooled brains, all species), avian avulavirus-1(previously known as avian paramyxovirus-1), avian influenza virus (tracheal/cloacal swabs, all birds), infectious laryngotracheitis virus (pooled tracheal swabs, gallinaceous birds), infectious bronchitis virus (pooled tracheal and pooled cloacal swabs, gallinaceous birds), infectious bursal disease virus (pooled cloacal swabs, chickens), fowl adenovirus (pooled cloacal swabs, chickens) and reovirus (pooled cloacal swabs, chickens and turkeys). PCR was also conducted for *Mycoplasma synoviae* (qualitative), *M. gallisepticum* (quantitative, pooled tracheal swabs for gallinaceous birds, and pooled airsac swabs for waterfowl), *M. meleagridis* (quantitative, pooled tracheal swabs, turkeys) and *M. iowae* (quantitative, pooled tracheal swabs, turkeys and gamebirds). *Mycoplasma* isolation was also conducted on pooled airsac swabs from waterfowl.

RESULTS

A total of 160 submissions, corresponding to a total of 246 birds, were received. Chickens were most common (134 submissions, 83.8 %) with layers as the

most common production type followed by broilers, dual purpose and breeders. There were 10 submissions (6.3%) of turkeys, 8 submissions (5%) of gamebirds, and 8 submissions (5%) of ducks.

Considering only the primary disease process for each bird (e.g., cause of death or clinical signs), a total of 248 final diagnoses were produced. The most common etiologic category / process was bacterial (23% of diagnoses), viral (14.5%, including Marek's disease [10.9%]), neoplastic (10%), nutritional/metabolic (8.9%), and parasitic (8%), including three cases of histomoniasis in turkeys and two cases of leukocytozoonosis in ducks. Mixed viral and bacterial infection causing multifactorial respiratory disease was tallied as a separate category, and constituted 15.7%. Pre-set microbiological tests detected *Campylobacter* spp., *Brachyspira* spp., *Mycoplasma synoviae*, *Mycoplasma gallisepticum*, and *Salmonella* spp. (*S*. Uganda, Anatum, Indiana, Montevideo, Ouakam) in 34.6, 33.3, 36.1, 27.1, and 3.1 % of tested submissions. Infectious bronchitis virus, fowl adenovirus, infectious laryngotracheitis virus, and reovirus were detected in 39.3, 35, 15.8 and 6.2% of tested submissions. Non-virulent avian avulavirus-1, determined to be lentogenic / vaccine virus was isolated from a 2.5-week-old heritage chicken submission (two birds), and avian influenza virus of wild bird origin was isolated from one two-week-old turkey poult (H10N8, LPAIV).

DISCUSSION

The most common submitting concern was that of increased mortality or morbidity. Multifactorial respiratory diseases and Marek's disease were common causes for the reported clinical signs. In contrast to other studies, prevalence of *Salmonella* spp. isolated from ceca using enrichment methods or from parenchymal organs using normal culture methods was low. As for federally important reportable diseases, there were no isolations of *Salmonella* Pullorum and *Salmonella* Gallinarum from any of the pooled ceca. One low pathogenic avian influenza virus, determined to be of wild bird origin, was isolated from a young turkey poult. This bird was one of multiple poults that had been recently purchased from possibly multiple vendors at a local fur and feather sale. Avian avulavirus-1 was detected from a submission of young heritage chickens from a newly assembled flock of young mixed heritage breeds, and was determined to be a lentogenic / vaccine strain.

Through the identification of common disease processes and the completion of a standardized questionnaire by small flock owners, analyses may reveal correlations between specific farming practices and the risk of certain infectious agents. These data will help to determine the need and scope for specialized training regarding poultry medicine and management aimed at Ontario veterinarians with an interest in small poultry flock medicine, as well as developing educational tools for small flock producers aimed at prevention and control of relevant diseases and contribute to provincial and federal disease surveillance.

ACKNOWLEDGEMENTS

This project was funded (or partially funded) by the OMAFRA-University of Guelph Strategic Partnership, under the Disease Surveillance Plan, which is a joint federal-provincial Growing Forward 2 project."

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

REFERENCES

1. Chicken Farmers of Ontario. Family Food Program. Welcome to the Chicken Farmers of Ontario Family Food Program. Available at: https://www.familyfoodgrower.ca/. Accessed May 31, 2017.

2. Buregeya JM, McEwen S, Neil J, et al. Biosecurity Practices and Geospatial Map of Ontario Backyard Poultry Flocks. *Journal of Environmental Science & Engineering* 2013:694–701.

3. Mainali, C., and Houston, I. Small Poultry Flocks in Alberta: Demographics and Practices. Avian Dis. 61:46–54. 2017.

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

5. Smith, E.I., Reif, J.S., Hill, A.E., Slota, K.E., Miller, R.S., Bjork, K.E., and Pabilonia, K.L. Epidemiologic characterization of Colorado backyard bird flocks. Avian Dis.56:263–271. 2012.

6. Madsen, J.M., Zimmermann, N.G., Timmons, J., and Tablante, N.L. Prevalence and Differentiation of Diseases in Maryland Backyard Flocks. Avian Dis.57:587–594. 2013.

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

8. Smith, G., Dunipace, S. How backyard poultry flocks influence the effort required to curtail avian influenza epidemics in commercial poultry flocks. Epidemics.3:71–75. 2011.

9. Crespo, R., Shivaprasad, H.L., Woolcock, P.R., Chin, R.P., Davidson-York, D., and Tarbell, D. Exotic Newcastle Disease in a Game Chicken Flock. Avian Dis. 43:349. 1999.

10. Capua, I., Dalla, P.M., Mutinelli, F., Marangon, S., and Terregino, C. Newcastle disease outbreaks in Italy during 2000. Vet Rec. 150:565–568. 2002.

11. Bavinck, V., Bouma, A., van Boven, M., Bos,M.E.H., Stassen, E., and Stegeman, J.A. The role of backyard poultry flocks in the epidemic of highly pathogenic avian influenza virus (H7N7) in the Netherlands in 2003. Prev Vet Med.88:247–254. 2009.

DETECTING ANTIBODIES AGAINST AVIAN INFLUENZA (AI) IN SYNANTHROPIC WILD BIRDS IDENTIFIED AS HIGH PRIORITY FOR AVIAN INFLUENZA TESTING IN NORTHERN CALIFORNIA

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ABSTRACT

While it is well understood that waterfowl are the primary reservoir of avian influenza viruses (AIv) there is much less understanding of how (ie. mechanically or via infection) avian "bridge" species (non-Anseriformes) can transmit the AIvs to commercial poultry. This is especially relevant for synanthropic birds that are known to be present near or inside poultry barns and are also known to share habitat with waterfowl. Species such as Brewer's Blackbird, European Starling and Red-Winged Blackbird fit this description and are therefore considered the "bridge" between the primary reservoir (ie. waterfowl) of AIv and commercial poultry. In order to help clarify the specific risk of these "bridge" species as a significant disease reservoir for commercial poultry in California, serum samples collected from synanthropic wild birds were tested for antibodies to AI. In particular, samples collected from areas with historically high waterfowl densities were tested. Using this criteria, 603 out of 69,451 sera samples were identified as high priority for AI testing.

INTRODUCTION

The 2014-2015 outbreak of highly pathogenic avian influenza (HPAI) resulted in the depopulation of over 48 million chickens and turkeys (10) and about $3.3 billion in economic losses (9). Additionally, while human infections have not been linked to HPAI outbreaks in the United States recently, AIv have infected humans in other parts of the world. More specifically, as of 30 October 2017, HPAI H5N1 has infected at least 860 individuals and caused 454 mortalities across 16 countries (14). Therefore, studying the main reservoirs of AIv, Anseriformes (ie. ducks, geese and swans) and Charadriiformes (ie. gulls, terns and shorebirds) (13), has become a priority particularly their migratory movement and their role in the persistence and transmission of AIv.

Waterfowl and shorebirds rarely interact with commercial poultry (2). In contrast, passerines have been observed near or inside poultry facilities (1, 4). In addition, passerines interface with waterfowl and/or their environment (i.e. wetlands, ponds) (1, 2). Therefore, passerines have been implicated as bridge hosts, which can facilitate the transmission of avian influenza (AI) by acting as a "bridge" between the natural maintenance host of AI (ie. waterfowl and shorebirds) and commercial poultry (7). Whether bridge hosts can transmit AI mechanically or by shedding it in their feces is not well understood.

In this study, historic sera from selected synanthropic wild passerines in California were tested for AI antibodies in order to better characterize the risk of AI transmission to commercial poultry from these "bridge" species.

MATERIALS AND METHODS

Based on a literature search and with the help of the United States Geological Service (USGS), Brewer's Blackbirds (*Euphagus cyanocephalus*), European Starlings (*Sturnus vulgaris*) and Red-Winged Blackbirds (*Agelaius phoeniceus*) were identified as potential bridge hosts of AI based on their behavior and habitat preferences relative to waterfowl. Out of 69,451 serum samples collected from various small perching birds across CA from 1996 to 2009,

1,555 serum samples were identified as coming from those selected species. NEXt Generation RADar (NEXRAD) was used to confirm overlap between waterfowl and the selected "bridge species."

Out of 1,555 sera samples, 603 samples were identified as "high priority" for AI testing and thereby selected for agar gel immunodiffusion (AGID) testing. Samples were tested AI antibodies by AGID at the California Health and Food Safety laboratory.

RESULTS AND DISCUSSION

While much of the literature on passerine birds indicate they are not reservoirs of AI, their role as intermittent hosts or fomites has been observed (8). In particular, starlings have been shown to be susceptible to AI (5, 7, 8) and sequencing analysis have implicated them as "bridge" hosts in Australia (6). In addition, previous field observations of European starlings indicate that their behavior may contribute to their susceptibility to AI (5). More specifically, starlings have been observed to congregate at high densities (ie. almost 530 per cubic meter) during roosting but then spread out during the day to search for food (5). This dispersal can increase their chances of coming in contact with AIv and their roosting behavior can help spread and maintain AIv strains in the flock and the environment. However, based on the literature it seems that variation in testing (ie. PCR, sequencing) and lack of validation (ie. virus isolation) limit the findings on passerines as "bridge" hosts for AIv (8).

While roosting and feeding behavior of starlings are strong indicators that starlings and birds that behave similar to them are more likely to have higher amounts of antibodies, the literature suggests that AI prevalence in captive and wild passerines is low at about 0.5% (8). Additionally, while antibody prevalence is about 6% for both captive and wild birds, AI antibody prevalence for wild birds alone is 0% (8). Nevertheless, it is important to keep in mind that these findings are for various passerines that may not necessarily be viable "bridge" hosts. Therefore, we tested Brewer's Blackbirds, European Starlings and Red-Winged Blackbirds, which have been identified as "high priority" for AI testing in California due to their strong likelihood of acting as bridge hosts (1, 4, 7, 12). In fact, European Starlings and Red-Winged Blackbirds have been observed near poultry barns and wetlands/ponds consistently in the literature (1, 4, 7). While the literature indicates Brewer's Blackbird do not go near barns as consistently as European Starlings and Red-Winged Blackbirds (1, 4), they have been identified as a "high priority" species in California (12).

CONCLUSION

Following the 2014-2015 outbreak of HPAI in the U.S., further insights regarding AI hosts and transmission are fundamental toward developing a better understanding of AI ecology. Non-waterfowl birds such as passerines should not be overlooked as important reservoirs or vectors of AI because of their potential to interface with both waterfowl and domestic poultry. Anecdotal and survey-based observations indicate it is not uncommon to see wild birds near barns or even inside barns (3, 11). In addition, one study calculated that indirect contact between the maintenance reservoir (ie. waterfowl) and the target species (ie. poultry) via a "bridge" host (ie. starling) was 20-fold more frequent than a direct contact between the main reservoir (ie. waterfowl) and the target species (ie. commercial poultry) (2). Therefore testing serum from species determined as "high priority" for AI testing in California could help clarify the overall risk of AI transmission to commercial poultry and help focus biosecurity efforts.

(The full-length article will be submitted to the Journal of *Avian Diseases*.)

REFERENCES

1. Burns, T. E., C. Ribble, C. Stephen, D. Kelton, L. Toews, J. Osterhold, and H. Wheeler. Use of observed wild bird activity on poultry farms and a literature review to target species as high priority for avian influenza testing in 2 regions of Canada. Canadian Veterinary Journal-Revue Veterinaire Canadienne 53:158-166. 2012.

2. Caron, A., V. Grosbois, E. Etter, N. Gaidet, and M. de Garine-Wichatitsky. Bridge hosts for avian influenza viruses at the wildlife/domestic interface: An eco-epidemiological framework implemented in southern Africa. Preventive Veterinary Medicine 117:590-600. 2014.

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

4. Houston, D. D., S. Azeem, C. W. Lundy, Y. Sato, B. Q. Guo, J. A. Blanchong, P. C. Gauger, D. R. Marks, K. J. Yoon, and J. S. Adelman. Evaluating the role of wild songbirds or rodents in spreading avian influenza virus across an agricultural landscape. PeerJ 5:24. 2017.

5. Lipkind, M., E. Shihmanter, and D. Shoham. Further characterization of H7N7 avian influenza-virus isolated from migrating starlings wintering in Israel. Zentralblatt Fur Veterinarmedizin Reihe B-Journal of Veterinary Medicine Series B-Infectious

Diseases Immunology Food Hygiene Veterinary Public Health 29:566-572. 1982.

6. Nestorowicz, A., Y. Kawaoka, W. J. Bean, and R. G. Webster. Molecular analysis of the hemagglutinin genes of Australian H7N7 influenza viruses: Role of passerine birds in maintenance or transmission? Virology 160:411-418. 1987.

7. Shriner, S. A., J. J. Root, M. W. Lutman, J. M. Kloft, K. K. VanDalen, H. J. Sullivan, T. S. White, M. P. Milleson, J. L. Hairston, S. C. Chandler, P. C. Wolf, C. T. Turnage, B. J. McCluskey, A. L. Vincent, M. K. Torchetti, T. Gidlewski, and T. J. DeLiberto. Surveillance for highly pathogenic H5 avian influenza virus in synanthropic wildlife associated with poultry farms during an acute outbreak. Sci Rep 6:11. 2016.

8. Slusher, M. J., B. R. Wilcox, M. P. Lutrell, R. L. Poulson, J. D. Brown, M. J. Yabsley, and D. E. Stallknecht. Are passerine birds reservoirs for influenza A viruses? J. Wildl. Dis. 50:792-809. 2014.

9. Swayne, D. E., R. E. Hill, and J. Clifford. Safe application of regionalization for trade in poultry and poultry products during highly pathogenic avian influenza outbreaks in the USA. Avian Pathol. 46:125-130. 2017.

10. United States Department of Agriculture, and Animal and Plant Health Inspection Service. Highly Pathogenic Avian Influenza Infected Premises 2014–2015. https://www.aphis.usda.gov/animal_health/animal_dis_spec/poultry/downloads/hpai-positive-premises-2014-2015.pdf. 2015.

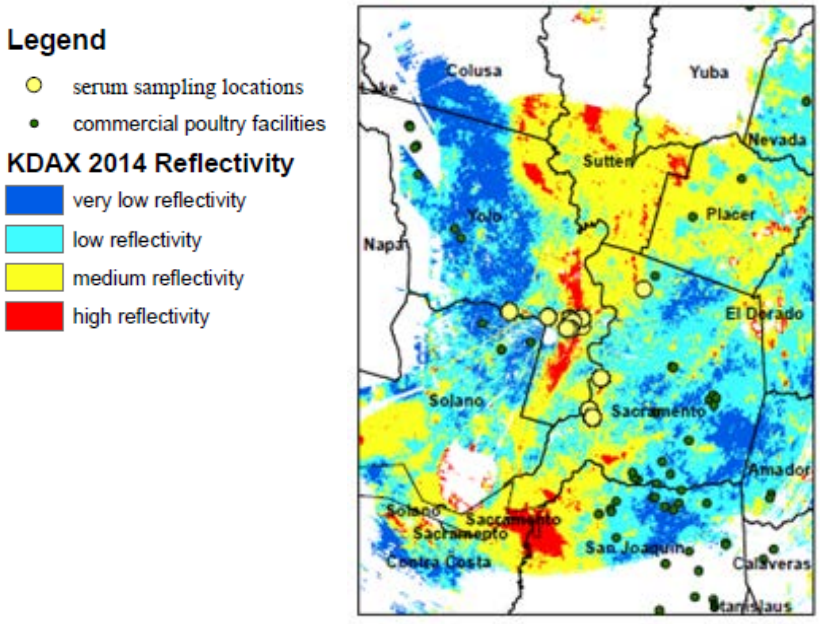
11. USDA-APHIS. Epidemiologic and Other Analyses of HPAI-Affected Poultry Flocks: July 15, 2015 Report. https://www.aphis.usda.gov/animal_health/animal_dis_spec/poultry/downloads/Epidemiologic-Analysis-July-15-2015.pdf. 2015.

12. USGS. Personal communication Elliott Matchett. 2017.

13. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. Evolution and ecology of influenza-A viruses. Microbiol. Rev. 56:152-179. 1992.

14. WHO. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO. http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/. 2017.

Figure 5. Map showing location of serum sample collection sites, commercial poultry facilities (<3k birds) and reflectivity from 2014 NEXRAD data with red and blue symbolizing high and low bird density, respectively.



SALMONELLA CONTROL PROGRAMS IN THE USA

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SUMMARY

As highlighted by the media in two recent outbreaks of *Salmonella enterica* serovar Heidelberg that sickened a substantial number of people, *Salmonella* contamination of poultry products remains a major public health concern for health authorities. In one of the outbreaks 430 people from 23 States and Puerto Rico were affected while in the other one 28 people from 13 States were affected. Every time an outbreak of *Salmonella* associated with consumption of poultry products occurs, it has a negative impact on the poultry companies involved as well as on the industry as a whole; therefore, controlling *Salmonella* has become a priority for many companies.

The Centers for Disease Control & Prevention (CDC) estimated in 2012 that 29% of food-borne outbreaks of salmonellosis in humans can be traced to the consumption of poultry products and that this constitutes the largest contributor among food products. For many years, the poultry industry through programs like the National Poultry Improvement Plan (NPIP) at the live production level or the Hazard Analysis of Critical Control Points (HACCP) programs at the processing plants has successfully diminished the rate of contamination in poultry products. Since table-eggs are known to be a documented source of human infections, particularly with *Salmonella enterica* serovar Enteritidis (over 80% of human outbreaks with identifiable sources have been attributed to this species), many Quality Assurance programs have been developed at the State and Federal level to reduce its incidence, monitor its prevalence, and minimize the possibility of contamination of shell eggs and the number of outbreaks in the human population. In spite of this, and as highlighted by a massive outbreak of *S.* Enteritidis in 2010 from two table-egg producers in Iowa that sickened nearly 2,000 people and resulted in the recall of over half a billion eggs, a great deal of work remains to be done if the impact of human food-borne *Salmonella* infections of poultry origin is to be minimized. In response to these type of outbreaks, the Food & Drug Administration (FDA) put in effect since July 9, 2010 the "Egg Safety Rule" to prevent the contamination of shell eggs with *S.* Enteritidis during production, storage and transportation. FDA stated that with its new rule it expected to prevent 79,000 illnesses and 30 deaths caused by *S.* Enteritidis-contaminated shell eggs each year.

In a similar way, and in order to minimize the risk of contamination of poultry meat with *Salmonella*, in July 2011 the Food Safety & Inspection Service (FSIS) implemented new performance standards for the maximum number of *Salmonella* positive chilled carcasses for broiler chickens and turkeys. And in a similar way, in February 2016 USDA-FSIS announced the finalization of new federal standards to reduce *Salmonella* and *Campylobacter* in ground chicken and turkey products as well as in raw chicken breasts, legs, and wings. Based on risk assessments, the USDA-FSIS estimated that the new standards will prevent 50,000 illnesses per year. In April 2017, USDA-FSIS published its Pathogen Reduction for *Salmonella* and *Campylobacter* that included Performance Standards Verification Testing.

Poultry producers have a variety of intervention strategies at their disposal at the production level (pre-harvest), during transportation (harvest) and at the processing plant (post-harvest) to minimize the risk of *Salmonella* contamination in the final product. These methods will be discussed with particular emphasis on the vaccination of broiler breeder and table-egg pullets as a major aid in *Salmonella* prevention programs.

EFFICACY OF COMPETITIVE EXCLUSION TO REDUCE SALMONELLA IN BROILER CHICKENS

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SUMMARY

This study aimed to to investigate the effects of competitive exclusion (CE) on *Salmonella* infection in broiler chickens. The protective effect of CE on *Salmonella* cecal colonization was evaluated in 1 day-old chicks. In laboratory trial, the CE products were administered by oral inoculation, drinking water and whole body spray. Three days after treatment, chickens were challenged with 10^7cfu/mL of *Salmonella* Enteritidis orally and were evaluated for 10 days. The CE-treated chickens showed comparable protection, and the number of *Salmonella* in the cecal contents was significantly decreased)P<0.05(compared with those of the positive control. Furthermore, the CE-treated chickens displayed greater intestinal histology, including villous height, submuscular layer and cell mitosis. These studies demonstrate that CE could protect chickens from *Salmonella* cecal colonization, and it is apparent that new methodologies associated with the development of a workable CE program are needed in the poultry industry.

INTRODUCTION

Salmonellosis is the most common and widely distributed foodborne disease. The main sources of *Salmonella* are poultry, meat and eggs. *Salmonella* accesses the food chain through the production of contaminated food)14(. Therefore, *Salmonella* control has become a major task in poultry production to ensure poultry food safety)2(. One of the most effective methods to control *Salmonella* is to treat young chicks with CE culture. The purpose of this study was to evaluate the efficacy of CE products against *Salmonella* cecal colonization in the intestine of chickens when administered CE orally, and by drinking water and whole body spray at one day old in a laboratory and at a farm.

MATERIALS AND METHODS

CE culture. The CE product was isolated from ceca of *Salmonella* free *broiler-grandparent* chickens at the age of 30 weeks. Each cecum was cut into a plastic bag to blend in the stomacher Lab Blender)Tekmar Co., Cincinnati, Ohio(containing 225 mL of normal saline. The serial dilution technique was performed, and then the dilution was spread onto Viande Levure agar)VL(plates modified from Sasipreeyajan and Saitanu)1994(and incubated at 42°C for 48 hr in a microbiological incubator)MIR253, Sanyo(. The CE product was submitted to be characterized by the National Science and Technology Development Agency)NSTDA(, Ministry of Science, Thailand. The mixture included three microbial strains in the CE product, including *Bacillus* spp., *Pediococcus acidolactici* and *Kodamaea ohmeri*. All microbes were tested for their safety and ability to inhibit the growth of *Salmonella*. For their safety, all microbes in the CE product were Gram's positive strains and lacked any important intestinal pathogen, including *Salmonella* and *Escherichia coli*, using selective media. Salmonella inhibition assay *in vitro* was performed using well diffusion assay)13(.

Briefly, Mueller Hinton agar plates inoculated with *Salmonella* and wells were drilled out using pasture pipettes 50 µL aliquots of cell free cultures supernatant in fresh VL broth of CE- culture suspended in the agar wells. Plates were incubated for 48 to 72 hr at 37°C until presenting inhibition zones around wells, which were interpreted as a positive result. To prepare the CE products inoculum, 1 mL of CE products was inoculated into 50 mL of VL broth and incubated for 18 hr at 42°C with shaking at 100 rpm.

***Salmonella* culture.** *Salmonella enterica* serovar Enteritidis obtained from the Department of Medical Science of Thailand was selected for its resistance to novobiocin and nalidixic acid in the laboratory and maintained on nutrient ager. For the experiment, *Salmonella* cultures were grown in tryptic soy broth)TSB(for approximately 18 hr with shaking at 100 rpm. The viable cell concentration of the inoculums was determined by colony count on xylose lysine deoxycholate agar)XLD agar(plates. *Salmonella* was stored separately and frozen at -80 °C in enrichment broth with glycerol)40%, w/v(until used.

Chickens and experimental designs. The *Salmonella* challenging model developed was modified using the methods of Sasipreeyajan and Saitanu (10). Forty, one-day-old mixed sex chickens of COBB500 were randomly divided into four groups of 10 chicks each: Group 1 was a positive control group; Group 2 was orally inoculated with 0.5 mL of CE products, containing approximately $1x10^7$cfu/mL, once a day for three days; Group 3 was provided CE products via drinking water at 1×10^7 cfu/mL for three days; Group 4 was sprayed with CE products using a whole body spray in a confined chicken shipping box at 1.75 mL/dose with a final concentration of 1×10^7 cfu/mL at 1-3 days of age. Feed and water were provided *ad libitum*, and chicks were observed regularly. Three days after CE application, all groups were challenged orally with 1×10^7 cfu/mL of *Salmonella* for three days. At 10 days of age, 10 chickens from each group were euthanized.Animal experiment protocols were performed according to the guidance and legislative regulations on the use of animals for scientific purposes of Chulalongkorn University, Bangkok, Thailand with permission no. 1431101.

Detection of *Salmonella* in the experimental chickens. In each experiment, chickens were euthanized. Ceca were collected aseptically and evaluated for *Salmonella* colonization by using serially dilution and *pour-plate* technique with XLD agar. The plates were incubated overnight at 37 °C. Colony morphology was used to differentiate bacterial types. The typical colonies appeared black or black-centered with a yellow periphery after 18-24 hr of incubation. Upon continued incubation, the colonies became entirely black or pink to red with black centers. *Salmonella* inhibition)%(was calculated by dividing the number of *Salmonella* negative chickens with the total number of *Salmonella* challenged chickens.

RESULTS AND DISCUSSION

Bacterial isolates from cecum of normal chickens were selected based on their ability to inhibit *Salmonella* growth *in vitro* and demonstrate that a culture consisting of LAB, *Bacillus* and yeast was efficacious *in vivo*. This finding supported the hypothesis of *Salmonella* reduction using CE product as previously described)6, 8, 11(. The experimental challenge dose of 10^7cfu/mL *Salmonella* resulted in cecal colonization in 100% of the 10-day-old positive control chickens)Table 1(. Additionally, the cecal of the control chickens contained 4.68×10^{10} cfu/mL of *Salmonella* per gram of content. In contrast, all CE treated groups revealed that *Salmonella* colony count was significantly lower than the positive control)P<0.05(. Particularly, the CE treated group via oral inoculation was significantly lower in the Salmonella colony count)P<0.05(compared to the others. For the percentage of *Salmonella* inhibition, the oral CE application group had the highest percentage)60%(compared to the others. In contrast, the positive control group showed 0% Salmonella inhibition. The amount of *Salmonella* population in the cecal contents of the body spray group was lower than in the drinking water group.

This demonstrated that CE products administrated by body spray produced better protection against *Salmonella* colonization compared with the drinking water administration. Several reports have revealed that the spray application of CE products to chickens at the hatchery is advantageous over drinking water treatment because it ensures early exposure to protective CE flora before any environmental *Salmonella* challenge in the rearing house)1, 4, 12(. The initial average BW of chickens was not different between groups)data not shown(. The average BW and FCR from the laboratory trial at 10 days of age are presented in Table 2. After challenge, the chickens in the positive control group had the lowest BW compared to the other groups)P<0.05(. Salmonella infections are mainly an asymptomatic disease in poultry, but it can be widespread and cause human illness)9(. Continuing interest to find a way to protect flocks from Salmonella infection and contamination of poultry products is needed.

In this study, the use of CE products as means to control infection or colonization of the gastrointestinal tract by *Salmonella* was performed. In the laboratory trial, statistical analysis revealed that at 10 days of age, the proportion of chickens with *Salmonella* infection in the CE-treated groups was lower than that of the control group)P<0.05(, meaning that the newly hatched chicks were protected against the establishment of *Salmonella* in the ceca when the CE product was provided. The LAB species of CE product can produce volatile fatty acid and lactate. Lactate will be converted to propionic acid, which has been shown to inhibit *Salmonella* colonization of the ceca and crop of chicks)7(. Effectiveness of CE products has been shown to accelerate development of normal microflora in chicks and poults, providing increased resistance to infection by *Salmonella* and some enteric bacterial pathogens)3(. In addition, CE products produce propionic acid when the level of cecal propionic acid increases, while the level of *Salmonella* found in the digestive tract of the chicken decreases or is eliminated)5(.

In the current study, chickens were challenged with a pathogenic strain of *S.* Enteritidis. The result revealed that all chickens had been successfully

infected. The infection was confirmed by determined average bacterial count of $4.68x10^{10}$ *Salmonella* in 1 mL of cecum contents, and also all 10 chicks showed positive infection of *Salmonella* in the control groups. In this experiment, the CE product was administered by oral inoculation, drinking water and spraying. Administration of the CE product via oral inoculation showed *Salmonella* recovery from the cecum to be significantly lowest. However, the administration of the CE product by oral inoculation is impractical compared to the spray application. The CE spraying group showed that *Salmonella* recovery from the cecum was significantly lower than the group that received the CE product in drinking water)P<0.05(. The administration of the CE product by spraying is more practical for use in hatcheries.

In the field trials, both control groups revealed *Salmonella*-positive around 10-20%, but no *Salmonella* was found in the CE-treated group.

REFERENCES

1. Blankenship L. C., J. S. Bailey, N. A. Cox, et al. Two-step mucosal competitive exclusion flora treatment to diminish salmonellae in commercial broiler chickens. Poul Sci. 72:1667-1672. 1993.

2. Chambers J. R., and J Gong. The intestinal microbiota and its modulation for Salmonella control in chickens. Food Res Int. 44: 3149-3159. 2011.

3. Higgins J. P., S. E. Higgins, J. L. Vicente, et al. Temporal effects of lactic acid bacteria probiotic culture on salmonella in neonatal broilers. Poul Sci. 86:1662–1666. 2007

4. Goren E., W. A. De Jong, P. Doornenbal, et al. Protection of chicks against salmonella infection induced by spray application of intestinal microflora in the hatchery. Vet Quart. 6: 73-79. 1984

5. Kubena L. F., J. A. Byrd, C. R. Young, et al. Effects of tannic acid on cecal volatile fatty acids and susceptibility to Salmonella typhimurium colonization in broiler chicks. Poult Sci. 80: 1293-1298. 2001.

6. Lee Y. J., M. S. Kang, Y. K. Woo, et al. Competitive exclusion against *Salmonella gallinarum* of *Salmonella enteritidis* infected chickens. Vet Sci. 2: 33-36. 2001.

7. Nisbet D. J., S. C. Ricke, C. M. Scanlan, et al. Inoculation of broiler chicks with a continuous-flow derived bacterial culture facilitates early cecal bacterial colonization and increases resistance to *Salmonella typhimurium*. Food Prot. 57: 12-15. 1994.

8. Radovcic E. P., and I. C. Grozdanic. Competitive exclusion against *Salmonella enterica* subspecies *enteric* serovars *Enteritidis* infection in chickens. Vet arhiv. 3: 141-152. 2003.

9. Revolledo L., A. J. P. Ferreira, and G. C. Mead. Prospects in salmonella control: competitive exclusion, probiotics, and enhancement of avian intestinal immunity. J Appl Poul Res. 15: 341–351. 2006.

10. Sasipreeyajan J, and K. Saitanu. Protection of *Salmonella* infection in broiler by competitive exclusion. Faculty of Veterinary Science. 90th Year Chulalongkorn Scholarship. 1994.

11. Schneitz C. *Competitive exclusion* in poultry-30 years of research. Food control. 16: 657-667. 2005.

12. Schneitz C., M. Hakkinen, L. Nuotio , et al. Droplet application for protecting chicks against salmonella colonisation by competitive exclusion. Vet Rec. 126: 196. 1990.

13. Sgouras D., P. Maragkoudakis, K. Petraki, et al. In vitro and in vivo inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota. Appl Environ Microbiol. 70: 518-526. 2004.

14. Thiermann A., E. Bonbon, J. Caetano, et al. Prevention, detection and control of *Salmonella* in poultry. Terrestrial Animal Health Code. Chapter 6.5. 2011.

COMPARISON STUDY OF VARIOUS LIVE SALMONELLA VACCINATION PROGRAMS ON PROTECTION AGAINST A *SALMONELLA* HEIDELBERG CHALLENGE IN SPF LEGHORNS

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INTRODUCTION

Live S*almonella* Typhimurium (ST) vaccination of young pullets helps reduce *Salmonella* colonization and shedding prior to giving inactivated vaccines. There are currently three live ST vaccines available on the market. The purpose of this study was to compare the three most likely adopted vaccination programs for their ability to reduce infections/loads after challenge with a virulent *Salmonella* Heidelberg (SH).

MATERIALS AND METHODS

Two hundred eighty SPF leghorns were placed into eight different isolators (35 per) at day of age. Two isolators were randomly selected to house one of four different ST vaccine treatments based on the following post hatch spray primer followed by a two week water booster programs: 1) Vaccines A/A, 2) Vaccines B/B, 3) Vaccines B/C and 4) No Vaccine. At four days of age, several chicks were removed from each isolator to culture spleens and cecas in order to measure vaccine recovery. At 50 days of age, all birds were challenged orally with SH at a target dose of 10^6 CFU per bird. One week later, birds were necropsied and spleens and cecas collected. The spleen and one cecal pouch were cultured by enrichment and the other cecal pouch was cultured using the MPN enumeration method. Body weights were recorded at 14, 21, 43, and 57 days of age.

RESULTS

Both hatchery-labeled vaccines, A and B, were recovered from 100% of the cecal samples at 4 days of age while spleen recovery was 94.4% and 87.5%, respectively. Both males and females receiving Vaccine B at hatch weighed significantly less (12.3% and 9.0%, respectively) than controls at 14 days of age (see Table). At 21 days of age, only males on Program B/B had significantly lower weights (13.9%) but both sexes were significantly lighter on Program B/C (16.8% and 18.6%, respectively) at 21 days. By 43 and 57 days of age there were no longer significant differences between vaccine treatments. There were no significant differences in any groups on *Salmonella* incidence in either spleens or cecas post challenge as controls had a very high "take" rate (29/30 positives). However, Programs A/A and B/C had the lowest geometric mean cecal counts (78 and 21, respectively) compared to B/B and Controls (1293 and 2322). Program B/C was significantly lower than B/B in cecal counts while A/A was nearly significantly lower (P=0.066) than B/B on super shedders (MPN>100) at 23% vs. 73%.

DISCUSSION

Live ST vaccines A and B both gave 100% "takes" at four days of age, confirming that these vaccines were successfully delivered via spray post hatch. Vaccine B caused a temporary depression of weight gain in both males and females in the first two weeks. This weight depression persisted for at least another week in males that were re-vaccinated with Vaccine B or in both sexes that were boosted with Vaccine C. In contrast, birds receiving Vaccine A always had similar weights to controls. The very high take rate of the SH challenge (97% positive spleens and cecas) and high average count may partly explain why most vaccinates were still positive for SH post challenge. Nonetheless, there were reductions in average cecal counts and "super shedders" seen in Programs A/A and B/C that were not demonstrated in birds on Program B/B.

Table 1. Post vaccination body weights and SH challenge culture results by live ST program.

Trx.	Live ST Vaccination Program		Day 14 BW(g)		Day 21 BW(g)		Enrichment % positive		MPN testing of ceca	
	Day 0	Day 14	male	female	male	female	spleen	ceca	GMT	% >100
01	ST Vax A	ST Vax A	144a	135a	232a	207a	73	87	78	23**
02	ST Vax B	ST Vax B	128b	122b	205b	207a	57	100	1,293	73
03	ST Vax B	ST Vax C			198b	171b	76	97	21*	19*
04	None	None	146a	134a	238a	210a	97	97	2,322	63

Values within a column that have a different letter are significantly different.
*Indicates a value that was significantly lower than T04.
**Indicates a value nearly significantly lower than T04 (p=0.0658).

LIVE ST VACCINATION TRIAL IN A BROILER COMPLEX DEMONSTRATES REDUCTIONS IN SALMONELLA PREVALENCE IN BIRD RINSE SAMPLES AT REHANG AND PARTS SAMPLES AFTER FURTHER PROCESSING

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INTRODUCTION

Live *Salmonella* Typhimurium (ST) vaccines are sometimes used as a live production intervention to help reduce Salmonella colonization and shedding in broilers. Carcass rinsate sampling at rehang is considered a good index of the *Salmonella* incidence and levels that are coming into the processing plant. With the recent implementation of parts testing standards by FSIS, this is another parameter worth tracking. The purpose of this study was to measure the impact of live ST vaccination on the frequency, load and serotypes recovered from carcass rinsates and the incidence of positive parts samples over the course of a growout cycle.

MATERIALS AND METHODS

A broiler complex in the Southeastern United States ran a week on/off trial for a total of 13 weeks including six weeks of Poulvac® ST vaccinated flocks. *Salmonella* vaccination was conducted by cabinet sprayer in the hatchery followed by a coarse spray field boost. Each week, three carcass rinsates were taken from several lots for *Salmonella* incidence, enumeration (using the MPN method) and serotyping. Percent positive rinsates were calculated as well as mean *Salmonella* counts and serogroup distribution. One part sample per day was also tested for *Salmonella*. Flock performance was also captured and compared.

RESULTS

The vast majority (94%) of Salmonellae recovered from rinsates fell into serogroups B and C. While ST vaccination resulted in 31.7% fewer positive rinsates, a greater reduction was seen in Group B isolates (50%) than in Group C isolates (25%). Mean *Salmonella* counts (with individual scores capped at 40) were 4.31 in controls compared to 3.53 in vaccinated lots. In addition, the percentage of "super shedders" (rinsates yielding an MPN ≥40) was higher in controls than in vaccinates (5.44% vs. 4.41%). Finally, 60 vaccinated farms performed 2.4 points better on adjusted feed conversion compared to 64 control farms.

DISCUSSION

Poulvac ST vaccinated birds tested positive 32% less often at rehang and 41% less often on parts sampling. That live ST vaccination resulted in greater Group B reductions than Group C may not be too surprising—ST is a Group B. Still, Poulvac ST reductions in Group C isolations have been noted in other previously published study reports (WPDC 2002, SCAD 2018). Vaccinated flocks also performed better in the field, suggesting that salmonella infections may exact a cost in production in addition to processing.

Table 1. Prevalence results of rinsate and part samples after enrichment testing.

Vaccine Treatment	Total Number Samples	% Positive Rinsates by Serogroups				Total Percent Positive	Parts Results	
		B	C	D	Other		Total # Samples	% Positive
Control	200	12.50%	20.00%	0.5%	1.0%	35.00%	33	24.24%
Poulvac ST	180	6.67%	15.00%	0.56%	1.67%	23.89%	21	14.29%

GIANT CELLS AND PLASMACYTE ATYPIA OF DUCK BLOOD (FRANK INDICATORS OF IMMUNOSUPPRESSION)

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SUMMARY

Solitary mononuclear giant cells of the circulation are not widely known outside of neoplasia. However, sufficient numbers occurred in commercial ducks to warrant description. They were detected during standard differential counts (SDC) of duck blood and appear to be of two general forms. Some resemble lymphocytes or plasmacytoid cells and others appear as monocytoid types. Therefore, it is the purpose to demonstrate the types of giant cells found in circulating blood. These occurred in four flocks of adult commercial strain ducks ranging in age from 50 – 59 weeks. Each flock appeared healthy and no duck showed outward signs of disease. However, egg production was average or good but fertility in three of four flocks was poor.

INTRODUCTION

Giant cells (GC) formed by fusion of monocytes or macrophages characterize infections or inflammation. Due to fusion, they are multinucleated (MGC) and commonly seen in granulomas and certain malignancies. MGCs form by aggregation of heterophils or macrophages degenerate into caesous substance. However, mononuclear GCs of the circulation were detected during SDC of duck blood. These rare cells appeared to be of two types, plasmacytoid or monocytoid. They were in normal ducks from healthy flocks aged between 50-59 wk. However, egg production was average or good but fertility in three of four flocks was poor.

MATERIALS AND METHODS

Blood samples, SDC, and microscopy. Venous blood (1 to 3 mL) was drawn into tubes containing EDTA anticoagulant and approximately 3µL was spread into monolayers on microscope slides. After air-drying and post fixing in EtOH, slides were stained using an in-house version of Wright's method followed by a brief secondary exposure to Giemsa. Duplicate 200 cell leukocyte counts were at 40x magnification. Giant cells were classified as lymphocytoid or monocytoid types depending on resemblance to standard sized cells.

Microscopy was with an Olympus CX-41 light microscope (Olympus America, Center Valley, PA) equipped with Plan N 40x, 0.65 numerical aperture (high dry) and Plan N, 1.25 numerical aperture 100x (oil) objectives. Cells were photographed at 100x by an Infinity-2 1.4 megapixel CCD USB 2.0 camera, and images captured by Infinity Analyze software (Release 6.5.2) (Lumenera, Inc. Ottawa, Ontario, CA).

RESULTS

A giant plasmacytoid cell (D ~ 15 µm) with irregular edges patchy cytoplasm containing clear vacuoles surrounding an eccentric nucleus is shown in Fig. 1 A. A rectangular giant monocytoid cell (13 x 9 µm) is at the left of Fig. 1 B. A plasmacytoid cell with an atypical nucleus ("Tau cell") (2) is at the right. Both cells are surrounded by a field populated by small-encapsulated bacteria (arrow). A second example of a giant rectangular monocytoid cell (17.6 x 14 µm) is at the top left of Fig. 1 C. The irregular polyhedral cytoplasm has a deep stained ectoplasmic edge. Clear vacuoles are within amphiphilic stained cytoplasm. A thrombocyte is attached to its right edge. A reactive lymphocyte (far right) and an atypical heterophil (bottom left) are nearby. All are on a background of mildly poikilocytotic erythrocytes. A third giant monocytoid (~ 13.5 μm^2) has an irregular polyhedral shape and patchy cytoplasm with a clear paranuclear space (Fig 1 D). The drakes of the flocks with mono/plasmacytoid atypia had an average TWBC of 65K +/- 5K and hens had an average TWBC of 95K +/- 6. There was microscopic evidence for a fungus, *Hemomycetes avium* (1) in many blood samples from these flocks.

DISCUSSION/CONCLUSION

Mononuclear GC were detected in blood samples of mature commercial ducks between 50-59 wk. They appeared to be of two general types resembling standard sized plasmacytes and monocytes. Some display features of both plasmacytes and monocytes. GC sizes were about 15 µm in diameter however shapes varied form round to irregular polyhedrons. Some had nuclei with multiple nucleoli suggesting polyploidy. These giants are not

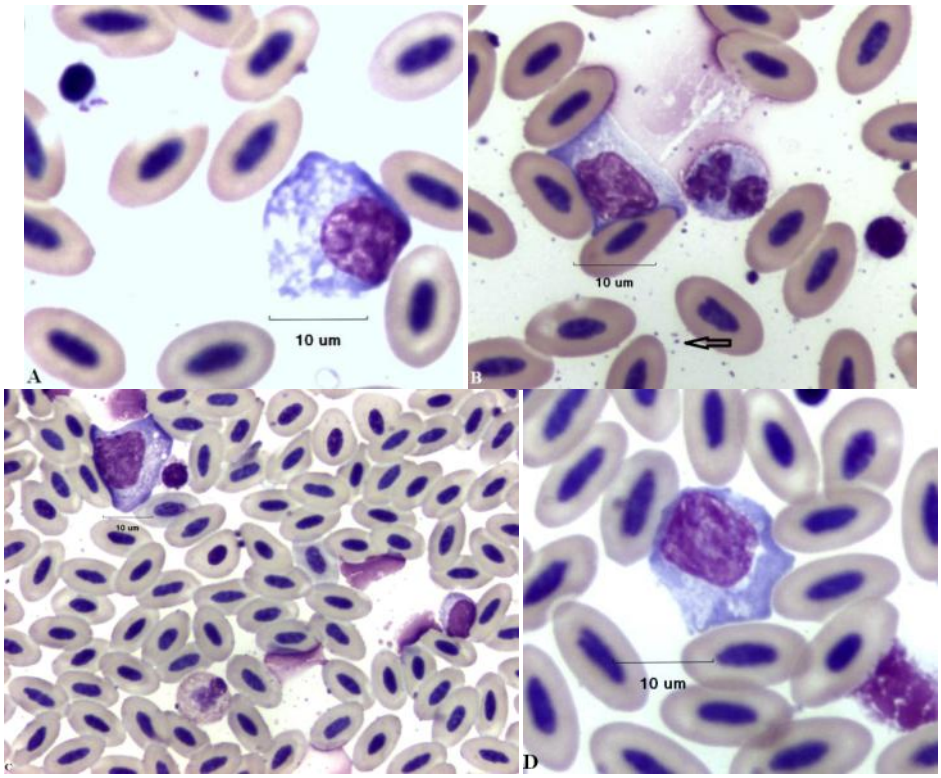
likely the result of fusions of diploid cells because they retain a single nucleus. Thus, they probably result by endomitosis without cytokinesis. They occur in samples with high TWBC, bacteria, and fungi and likely a consequence of microbial stimuli.

REFERENCES

1. Cotter, P. F. 2015. Hemomycetes avium, a blood-borne fungus of three poultry species. Abs. 293 Poult. Sci. Vol. 94 (E-suppl. 1) 101.

2. Cotter, P. 2016. Inflammation hematology of ducks illustrates the monocyte-macrophage continuum Abst. 238 Poult. Sci. 95(E-Suppl. 1) 81.

Figure 1. A. A plasmacytoid giant cell. B. A giant monocytoid, a Tau plasmacyte, and bacteria (arrow). C. A giant monocytoid attached by a thrombocyte, an atypical heterophil, and a reactive lymphocyte. D. A giant mono/plasmacytoid cell.



INFLAMMATORY HEMOGRAMS OF COMMERCIAL TURKEYS

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SUMMARY

Standard differential counts of venous blood from 10 week turkeys housed in pens indicated leukocytosis and complex inflammatory hemograms. Heterophils sub-classified on size were of four types ranging from dwarfs to giant cells. Tinctorial heterophil variants and toxic types were common. Additionally many dysplastic basophils and atypical eosinophils accompanied the heterophils. Atypical lymphocytes included giant cells and cells with Mott features (plasmacytes). Fragile giant monocytes were also found. Bacteria and fungi were in many of the smears, and these are the likely stimuli for the atypical hematology.

INTRODUCTION

As infectious morbidity and mortality account for a portion of the ~11% annual losses of turkeys grown in the USA, the study of their blood should provide valuable insight. It is the purpose to describe variation of turkey leukocytes with emphasis on atypical granulocytes and inflammatory lymphocytes because these cells are infection indicators. The TWBCs of toms in a study group was ~50K but the TWBCs of the hens was > 100K. Heterophil diameter size means sorted into four classes: dwarfs (6.5 μm) standard (7.7 μm) large (9.4 μm) to giant cells (12.4 μm). In addition oncosis (swollen) cells and tinctorial variants, and toxic cells were also detected. Hemograms also contained atypical (dysplastic) basophils (1) and atypical eosinophils having faintly stained cytoplasmic granules. Giant plasmacytoid lymphocytes (>12 μm) some binuclear, others with Mott cell features of cytoplasmic vacuoles (Russell bodies) were seen in the company of atypical heterophils and myelocytes. Giant monocytes were also common. As some of these fragile cells were partially disintegrated, they would ordinarily escape notice. All atypical leukocytes occurred in smears with bacteria and fungi, likely causes of leukocytosis. In conclusion, as most of these atypical cells have not been reported previously this study constitutes a first report. Collectively atypia as described indicate both immunosuppression and inflammation.

MATERIALS AND METHODS

Turkeys, Blood, Stain Procedure and Microscopy. Whole blood was drawn from the leg veins of 10 week turkeys housed in pens. Staining was by an in-house version of Wright's method followed by a brief secondary exposure to Giemsa. An eosinophil stain was with a brief preliminary exposure to Diff-Quik® fixative and "solution I" and followed by Wrights stain. Photos were obtained with an Olympus CX-41 light microscope with 100x oil objective; image capture with Infinity-2 1.4 megapixel CCD USB 2.0 Camera. Photo processing was with Infinity Analyze software.

RESULTS

Examples of normal heterophils, an atypical eosinophil and giant plasmacytes are in Fig. 1 A. Highly atypical heterophils and a giant cell with duel monocyte/plasmacyte features are in Fig. 1 B. The improved eosinophil staining using a duel exposure to Diff-Quik and Wright's stain is seen by comparing the cell in Fig 1 C with the eosinophil of Fig 1 A.

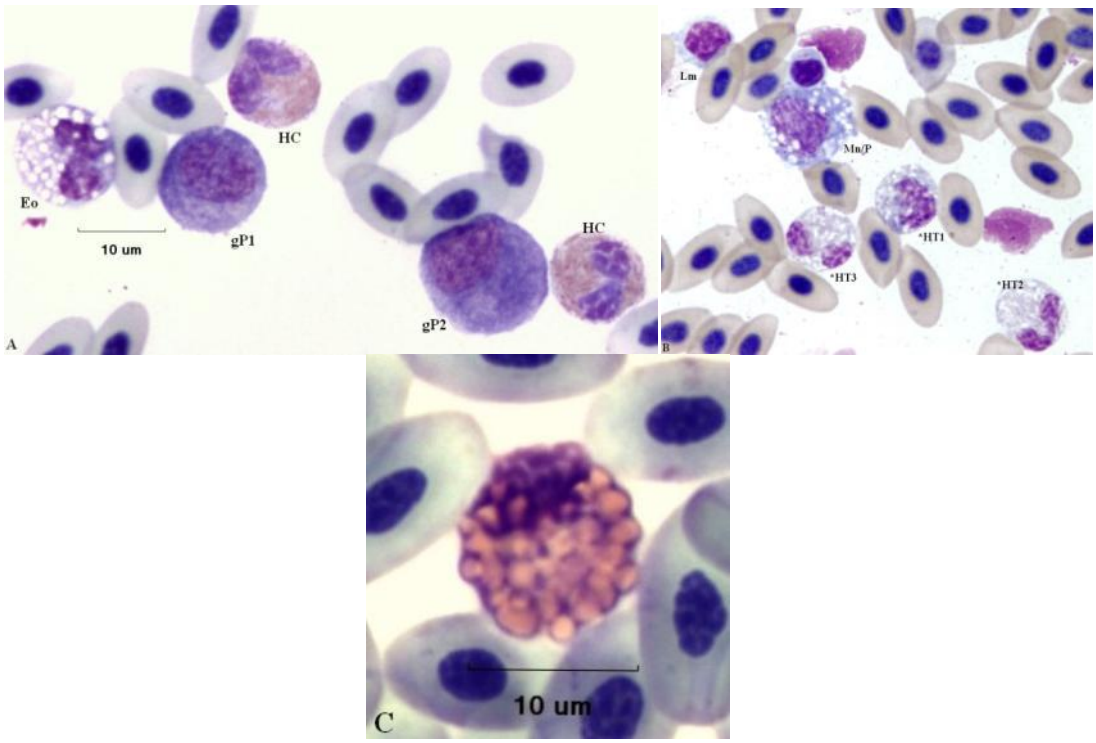
DISCUSSION

Atypical cells of turkey blood are indications of inflammation. Giant plasmacytoid cells and dysplastic heterophils are indicators of a "left-shift" likely the result of bacteremia and fungemia and often appear in the company of atypical basophils (1). Cells as these are usually found in smears also having very high TWBCs. No earlier reports of similar cells appear in the avian hematology literature. Eosinophil cytoplasmic granules are enhanced by the Diff-Quik/Wright procedure.

REFERENCE

1. Cotter, P. F., 2017. Basophilia and basophiliosis in caged hens at 18 and 77 weeks. Int. Jour. Poul. Sci.16 (2):23-30.

Figure 1 A. A field with an eosinophil (Eo) classic heterophils (HC) and giant plasmacytoid cells (gP1, gP2) with eccentric nuclei, coarse chromatin, and patchy paranuclear Hofs. **B**. Atypical heterophils with weakly stained (dysplastic) granules *HT. HT1 is an early developmental stage; HT2 andHT3 are a band stage and a two-lobe stage. Mn/P is a giant cell displaying monocyte and plasmacyte features. A reactive lymphocyte (Lm) is at the top left. **Figure 1 C** is an example of an eosinophil stained by Diff-Quik/Wright procedure; compare with Figure 1 A.



OUTBREAKS OF *ERYSIPELOTHRIX RHUSIOPATHIAE* IN LAYING HENS

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SUMMARY

The bacterium *Erysipelothrix rhusiopathiae* can infect a wide range of mammals and birds. Vermin are a significant risk factor for introducing infection into a flock. Among farm animals, pigs and sheep are most commonly infected. Turkeys are the most important domestic poultry species affected. Furthermore mature turkey flocks appear to be more susceptible to infection. Although it is known that some turkeys may serve as reservoirs of the bacteria, evidence of possible carrier state in chickens are scarce. However, *E. rhusiopathiae* has been isolated from healthy broiler chickens at slaughter, suggesting that chickens may be a potential reservoir of the infection. *E. rhusiopathiae* has been isolated from skin, feathers and larynx of healthy chickens, mainly in farms where chickens were raised alongside of pigs. Still erysipelas outbreaks are considered unusual in chickens.

Two cage free commercial layer flocks experienced three to four times increase in mortality and a 2% to 3% drop in egg production. The first outbreak occurred in 2016 when the flock was 75-week-old. The second outbreak occurred one year later, in a different house of the same complex when the birds were 40 weeks of age. At the time of the first outbreak, the flock did not have access to the outdoors, due to the threat of avian influenza from wild birds. Both flocks were vaccinated using the standard program for the company.

Six and 16 carcasses were submitted respectively to the Avian Health and Food Safety Laboratory. The most common gross finding in both cases were hepatomegaly (in 5/6 birds submitted and 15/16) splenomegaly (2/ 6 and 8/16), and free yolk in the celomic cavity (5/6 and 15/16). In the first case, excess mucus in the trachea without hemorrhage was observed in three birds. In the second case, mild northern fowl mite (*Ornithonyssus sylviarum*) infestation was observed in all the submitted birds and swollen kidneys were noted in 15 birds. Histologically, multifocal areas of necrosis with intralesional bacteria were present in the liver and spleen. Additionally, there was accumulation of fibrin and infiltration of heteterophils and macrophages in the lungs and kidneys. *E. rhusiopathiae* was isolated from the liver, abdomen, and ovary. Both flocks were negative for Hepatitis E virus. The first flock was weakly positive for infectious laryngotrachetis (ILT) by PCR only. The second flock was weakly positive for infectious bronchitis virus and negative for ILT by PCR.

Because the first flock was 75 weeks of age when the outbreak occurred the company did not treat the birds and the house was depopulated. At the time of writing this paper, the second flock is still on the farm. The house is kept under quarantine with strict biosecurity to avoid infecting nearby houses. The house is checked several times throughout the day and dead birds are removed promptly. The flock has been treated twice with bacitracin methylene disalicylate (BMD) in the water. Egg production in the flock continues to be below the expected and mortality is three times higher then expected.

It is still unknown how the bacterium was first introduced to the farm. It is suspected that *E. rhusiopathiae* can survive for more than one month outside the host, especially in soil contaminated by previous livestock. It has been known for erysipelas to recur on a site that has not been used for livestock for several years. However no pigs or sheep have ever been on the premise. Once *E. rhusiopathiae* is introduced, it may spread between poultry houses by contaminated manure and dust (3).

It is still not fully understood how the infection spreads between birds within a laying hen flock, although it has been suggested the bacteria passes through broken skin and mucous membranes, and that feather pecking and cannibalism may favor transmission (1). Experimentally, chickens have been infected both intramuscularly and orally (6). Flocks in free-range systems appeared to be at a higher risk than flocks in indoor litter based systems, while flocks in cages appeared to be at the lowest risk (5). Drinking water may be the source of contamination between birds from the same flock (3). Additionally, *Dermanyssus gallinae* (red mite) collected from affected flocks was shown to carry *E. rhusiopathiae* externally and internally, but the reservoir potential of the mite could not be proven (2, 4). On the other hand,

E. rhusiopathiae has never been isolated from northern fowl mites.

Because of the increasing trend for cage free flocks and lack of antibiotics, knowledge of environmental risks to better prevent outbreaks needs to be investigated. Using vaccination is one way to prevent erysipelas in flocks at risk. However, the procedure requires training and it is labor intensive because it involves subcutaneous injection in the leg fold.

In summary, outbreaks of erysipelas are uncommon in chickens, but because there is an increase in the number of cage free flocks it is likely outbreaks will occur more frequently. Lack of antibiotics to treat the disease requires better prevention methods, such as tight biosecurity and vaccination. Environmental sampling may help determining high risk areas on the farm.

REFERENCES

1. Bricker, J. M., and Y. M. Saif. Erysipelas. In: Diseases of Poultry, 13th ed. D. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez and V. Nair, eds. Wiley-Blackwell, Ames, IA. pp 909-922. 2013.

2. Chirico, J., H. Eriksson, O. Fossum, and D. Jansson. The poultry red mite, Dermanyssus gallinae, a potential vector of Erysipelothrix rhusiopathiae causing erysipelas in hens. Medical Veterinary Entomology 17:232-234. 2003.

3. Eriksson, H., E. Bagge, V. Båverud, C. Fellström, and D. S. Jansson. Erysipelothrix rhusiopathiae contamination in the poultry house environment during erysipelas outbreaks in organic laying hen flocks. Avian Pathol. 43:231-237. 2014.

4. Eriksson, H., S. Brännström, H. Skarin, and J. Chirico. Characterization of *Erysipelothrix rhusiopathiae* isolates from laying hens and poultry red mites (*Dermanyssus gallinae*) from an outbreak of erysipelas. Avian Pathol. 39:505-509. 2010.

5. Eriksson, H., A.-K. Nyman, C. Fellström, and P. Wallgren. Erysipelas in laying hens is associated with housing system. The Veterinary Record 173:18-21. 2013.

6. Mazaheri, A., M. Lierz, and H. M. Hafez. Investigations on the pathogenicity of *Erysipelothrix rhusiopathiae* in laying hens. Avian Dis. 49:574-576. 2005.

BACTERIAL DIVERSITY IN THE FECES OF WILD BALD EAGLES, TURKEY VULTURES, AND COMMON RAVENS FROM THE PACIFIC NORTHWEST COAST

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SUMMARY

Birds harbor diverse microorganism in their guts, which collectively fulfill important roles in providing them with nutrition and protection from pathogens. Although numerous studies have investigated the presence of certain pathogenic bacteria in the feces of wild birds few explore the range of intestinal microbiota of wild birds. Furthermore, these studies have analyzed gene sequences to the phylum or class level, with no attempt to understand these microbiota at the species level. In this study, we report on microbiota collected from the fecal samples of 21 avian scavengers captured on coastal beaches of Washington and Oregon between 2013 and 2015: ten turkey vultures (*Cathartes aura*), nine bald eagles (*Haliaeetus leucocephalus*), and two common ravens (*Corvus corax*).

All samples were sent to MR DNA (www.mrdnalab.com, Shallowater, TX) for analysis. The16S rRNA gene V4 variable region was performed on a MiSeq system. Sequence data were processed using a proprietary analysis pipeline. Final operational taxonomic units were taxonomically classified using BLASTn against a database derived from RDPII http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov).

Among notable pathogenic bacteria, sequence genes of *Salmonella enterica* were detected in all the fecal samples and accounted for 1.2-1.8% of all sequences. Interestingly, the prevalence of *S. enterica* in wild raptors' feces is reported between 1% and 20% by culture. *Chlamydia psittaci* sequences were detected in all fecal samples, but the prevalence was low (0.01% or less of all sequences), except in a turkey vulture sample where it accounted for 13.4% of all sequences. *Escherichia coli* was also detected in all three avian scavengers sampled, but sequences were sparse and detected in only a few fecal sample. *Clostridium perfringens* was one of the most common species sequences in turkey vultures, but represented less than 0.5% of the sequences detected in bald eagles and ravens. *Campylobacter* spp. sequences were detected in all fecal samples, but no *C. jejuni* genes were found. No *Pasteurella* spp. or *Mycoplasma* spp. sequences were detected.

The study demonstrated that the fecal flora vary among these three scavenger species even though they live in the same region and likely consume the same types of foods. This normal microbiota data will aid our understanding and interpretation of nutritional and intestinal disorders in these bird species. Identification and characterization of microorganism populations can be used as part of epidemiological, pathogen detection, and microbial diversity studies.

INFECTIOUS CORYZA OUTBREAK IN COMMERCIAL BROILERS IN CALIFORNIA: DIAGNOSTIC OVERVIEW

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SUMMARY

During 2017 the California Animal Health and Food Safety Laboratory System (CAHFS) Turlock branch, received a total of 38 submissions of commercial broiler chickens with a history of respiratory signs, increased mortality and poor growth performance. Neurological signs, including torticollis and disorientation, were also described in one case. Swollen-head like syndrome, accumulation of mucoid/caseous exudate within infraorbital sinuses and polyserositis were the most significant findings observed at necropsy. Microscopically, inflammation of the upper and lower respiratory tract was commonly observed, followed by facial cellulitis and polyserositis. In the majority of cases *Avibacterium paragallinarum* was isolated from upper and lower respiratory tract and confirmed by PCR method. In a small percentage of submissions, the bacterium was also recovered from heart, cranial bone and brain. Concurrent infectious agents detected by molecular testing included: infectious bronchitis virus, an endemic strain of infectious bursal disease virus and *Mycoplasma synoviae*. An overview of the outbreak from a diagnostic point of view will be provided.

INTRODUCTION

Avibacterium paragallinarum is a Gram-negative bacterium responsible for an acute respiratory disease in chickens, pheasants, guinea fowls, and Japanese quails. It is also known as infectious coryza. This condition has been associated with significant economic losses, in both meat-type and egg-type chickens, due to poor growth performance, increased condemnation rates at the processing plant and drop is egg production (1,2,5). Infectious coryza is typically an upper respiratory infection and transmission is usually mediated by direct or indirect contact with symptomatic or carrier birds (2,5). Unusual outbreaks of infectious coryza have been reported in commercial poultry production operations in different parts of the world (1). In particular, a swollen-head-like syndrome has been described in North and South America (5,9). Septicemia and arthritis have been observed in broilers and layers flocks in Argentina (9).

MATERIALS AND METHODS

During 2017, the Turlock branch of CAHFS, received a total of 40 submissions of commercial broiler chickens with a history of respiratory signs, increased mortality and poor growth performances. Submitted chickens ranged from 20 to 44 days of age. Live birds were humanely euthanized and a complete necropsy was performed on all submissions. Tissue sections were collected for histopathology and processed using hematoxylin and eosin staining. Tracheal swab pools were collected and tested for infectious bronchitis virus (IBV) by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (3) and for *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) by real time polymerase chain reaction (qPCR). Bursa of Fabricius tissue pools were also collected and tested for infectious bursal disease virus (IBDV) and very virulent IBDV (vvIBDV) by qRT-PCR (6). Virus isolation in embryonated chicken eggs was performed on 14 tracheal samples positive for IBV by qRT-PCR. The allantoic fluid from inoculated eggs was evaluated for the presence of IBV by qRT- PCR targeting the S1 gene of the viral genome and sequence analysis was performed (7). Avian influenza (AI) RT-PCR was also performed on oropharyngeal swab pools obtained from all cases. Selected organs were cultured for aerobic bacteria and plated onto 5% sheep's blood agar, MacConkey agar, and chocolate blood agar plates. Staphylococcus aureus nurse cultures were cross–streaked onto both blood agar and chocolate blood agar plates. All plates were incubated at 37ºC and 7% CO2 and examined at 24 and 48 hours post-incubation. Colonies suggestive of *A. paragallinarum* were confirmed by PCR method (4). Blood samples

were collected and sera were tested for Newcastle disease virus, AI, IBV, IBDV, MG, MS, and avian reovirus by enzyme-linked immunosorbent assay (ELISA). Plate agglutination test for MG was also performed.

RESULTS AND DISCUSSION

The majority of birds appeared depressed, reluctant to move and showed clinical signs indicative of a respiratory infection, including dyspnea, rales, coughing and sneezing. Palpebral edema, watery eyes and nasal discharge were commonly observed. Neurological signs, including torticollis, opisthotonos and disorientation were present in one submission. Swollen-head like syndrome, accumulation of mucoid/caseous exudate within infraorbital sinuses and polyserositis were the most significant findings seen at necropsy. Avibacterium paragallinarum was isolated from the upper and lower respiratory tract, including infraorbital sinus, air sac and lung, and confirmed by PCR method. In a small percentage of cases, the bacterium was also recovered from heart, cranial bone and brain. Other bacteria recovered from the respiratory tract included *Escherichia coli*, *Ornithobacterium rhinotracheale*, *Enterococcus faecalis*, and *Gallibacterium anatis* biovar haemolytica. Microscopically, mild to severe inflammation of the upper and lower respiratory tract was a constant finding, followed by facial cellulitis and polyserositis. Cranial osteomyelitis, otitis media and interna and meningoencephalitis were also present in a few cases. IBV was detected from tracheal swab pools by qRT-PCR in 24 cases. The IBV was also isolated by viral isolation technique in 12/14 cases. In addition, 1/8 tracheal swab pools was positive for MS by qPCR and 16/20 bursa tissue pools were positive for an endemic strain of IBDV by qRT-PCR. Serology showed positive antibodies titers for IBV, IBDV and avian reovirus. Several reports underlie the role played by other pathogens in unusual outbreaks of infectious coryza, including IBV, MG, MS, *Gallibacterium anatis* biovar. hemolytica, *Pasteurella* spp. and *Salmonella* spp. (1,2,5,8, 9)

Our diagnostic findings suggest that a variable combination of viral and bacterial co-infections resulted in an exacerbation of the clinical signs associated with infectious coryza in young broilers. In addition, the pathogenicity of *A. paragallinarum* can also vary according to the serovar involved (10,11) and further work is in progress in order to characterize the isolates obtained from this outbreak.

ACKNOWLEDEMENT

Molecular testing for avian influenza virus, infectious bronchitis virus and infectious bursal disease virus and viral isolation were done at the Davis branch of CAHFS.

(A complete manuscript will be submitted to *Avian Diseases*.)

REFERENCES

1. Blackall P. G. Infectious coryza: overview of the disease and new diagnostic options. Clin. Microbiol. Rev. 12:627-632. 1999.

2. Blackall P. G., and E. Soriano-Vargas. Infectious Coryza and related bacterial infections. In: Disease of Poultry, 13th ed. D. E. Swayne. Wiley & Sons Inc. pp. 859-873. 2013.

3. Callison S. A., D. A. Hilt, T. O. Boynton, B. F. Sample, R. Robison, D. E. Swayne, M. W. Jackwood. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J. Virol. Methods. 138:60-65. 2006.

4. Chen X, et al. Development and application of DNA probes and PCR tests for Haemophilus paragallinarum. Avian Dis. 40:398-407. 1996.

5. Droual R., A. A Bickford, B. R. Charlton, G. L. Copper, and S. E. Channing. Infectious coryza in meat chickens in the San Joaquin Valley of California. Avian Dis. 34:1009-1016. 1990.

6. Jackwood DJ, Sommer SE. Molecular studies on suspect very virulent infectious bursal disease virus genomic RNA. Avian Dis. 49:246-251. 2005.

7. Lukert PD. Infectious bronchitis virus. In: Hitchner SB et al, eds. Isolation and identification of avian pathogens. 2nd ed. Kennett Square, PA: American Association of Avian Pathologists, pp. 70-72. 1980.

8. Paudel S, et al. Coinfection of Avibacterium paragallinarum and Gallibacterium anatis in specific-pathogen-free chickens complicates clinical signs of infectious coryza, which can be prevented by vaccination. Avian Dis. 61:55-6. 2017.

9. Sandoval VE, et al. Complicated infectious coryza outbreaks in Argentina. Avian Dis. 38:672-678. 1994.

10. Soriano VE, et al. Virulence of the nine serovar reference strains of Haemophilus paragallinarum. Avian Dis. 48:886-889. 2004.

11. Trujillo-Ruiz HH, et al. Virulence of serovar C-1 strains of Avibacterium paragallinarum. Avian Dis. 60:837-840. 2016.

STUDIES ON THE INTERACTION OF NECROTIC ENTERITIS SEVERITY AND *SALMONELLA* PREVALENCE IN BROILER CHICKENS

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INTRODUCTION

Over the recent years the Food Safety and Inspection Service (FSIS) has tightened the *Salmonella* spp. compliance guidelines for the poultry industry. Concurrently, several broilers integrators have turned the page of their production systems and welcomed a non-antibiotic era. In 2003, a study evaluated and discussed the role of feed antibiotic removal on food safety in various commercial studies (3). Based on industry data collected over a five year period (32,000,000 chickens), the author concluded that antibiotic free flocks had more disease conditions and were highly related to an increased prevalence of *Salmonella* spp. (4). The European antibiotic ban example can be considered another good indicator of how antibiotic removal can result in collateral increase of food borne pathogens. Even though *Salmonella* spp. would not be expected to be affected by the ban of gram-positive spectrum antibiotics, it was observed that in some countries (e.g., Denmark) the cases of microbiologically confirmed *Salmonella* infections increased in prevalence (1). With this background, the objective of the following three broiler trials was to evaluate the effect of necrotic enteritis (NE) and respective intervention strategies on *Salmonella* Heidelberg (SH) prevalence and load.

MATERIALS AND METHODS

Three broiler pen studies were conducted to evaluate the effect of NE and respective intervention strategies on *Salmonella* prevalence. Each trial had 48 pens with 32 birds per pen grown up to 42 days of age. The NE challenge methodology was common across studies and consisted of an oral gavage of *Eimeria* spp. at 14 days followed by a *Clostridium perfringens* challenge at 18 days. The first study evaluated the effects of different times of exposure to SH (4, 18, and 21 days) on *Salmonella* prevalence in the presence of NE. Additionally, the effects of BMD as a NE intervention control were evaluated. Studies 2 and 3 had the objective of comparing BMD, zoalene and a commercially available probiotic as NE interventions on the prevalence of *Salmonella* when birds had a co-infection of NE and SH at 18 days of age. All of the SH challenges consisted of each bird receiving 0.5 mL of a 1.0x108 CFU/mL culture by oral gavage. Performance up to 42 days and NE severity three days post-challenge were evaluated. *Salmonella* prevalence was assessed in spleens, cecal pouches, carcass rinses, and drag boot swabs. In addition, SH enumeration was performed in cecae and boot swab samples. A P-value ≤ 0.05 was deemed statistically significant for all hypothesis testing with two-sided tests. Multiple comparisons among treatments were made using the Shaffer simulated methodology.

RESULTS

Performance. Across the three studies, feed conversion ratio (FCR) was significantly reduced by BMD at the end of the grow-out period. In addition, zoalene resulted in improved FCR when compared to the control and probiotic treatments in Studies 2 and 3, respectively. Body weight gain was similarly affected at 28 days by the feed additives.

Necrotic enteritis severity. Birds fed BMD had lower NE severity lesion scores when compared with other treatments across the three studies. In addition, BMD resulted in lower post NE challenge related and overall mortality. BMD fed birds had post challenge mortality rates lower than 0.8% whereas the other treatments had values ranging in between 21.1% and 32.1%.

***Salmonella* incidence and load**. In Study 1, birds that were co-infected with NE and SH at day 18 had higher recovery of salmonella from spleens, bootie swabs, and carcass rinses. Focusing on the 18 day SH challenge data, BMD resulted in a significant reductions ($P < 0.05$) of *Salmonella* load on bootie swabs (Control = 2.8x105 CFU/g vs. BMD = 8.2x102 CFU/g) and SH prevalence in carcass rinses (Control = 76% vs. BMD = 33%). Additionally, a BMD effect on reduction of SH prevalence in spleens was nearly significant (P=0.090).

There were no significant statistical differences between the different treatments on SH prevalence and enumeration in studies 2 and 3 (Table 1). However, BMD showed a consistent numerical trend on

reducing SH prevalence in all the samples evaluated. In Study 2, BMD resulted in 10% and 20% less positives on cecae and boot swabs than the controls. Further, enumeration values were 62% and 76% lower for cecae and boot swab samples, respectively. Likewise, zoalene and probiotic resulted in an overall SH reduction when compared to the controls, although to a lesser extent than BMD.

DISCUSSION

The ameliorative effects of BMD on performance and NE severity are well recognized in the literature (2) and were confirmed herein. Over the three studies, BMD resulted in FCR improvements between 3 to 16 points, extra weight gain up to 106 g, and a 21% to 32% NE post-challenge mortality reduction. The non-antibiotic interventions evaluated in Studies 2 and 3 did not show a beneficial effect on performance or mortality in the presence of NE challenge. Likewise, the severity of NE lesions was only reduced by the BMD intervention.

Regarding the effects of BMD on *Salmonella* recovery, it was noticeable in Study 1 that BMD significantly reduced SH prevalence and load (spleens, boot swabs and carcass rinses). This effect was especially evident when SH was co-infected with the NE challenge (18 days). Using a day old SH challenge and an analogous NE challenge model, a study evaluated the effects of BMD and roxarsone on *Salmonella* prevalence in broiler chickens (2). Contrarily, to what was observed in Study 1, the results showed that only roxarsone resulted in lower SH prevalence with no BMD effect present (2). The authors concluded that SH prevalence can be significantly reduced by *E. tenella* (roxarsone effect). Considering the results of the previous study and Study 1, it was concluded that strategies that counteract enteric disease can have an indirect effect on *Salmonella* recovery. With that in mind, Study 2 and 3 were devised to evaluate the effects of various enteric disease interventions on *Salmonella* prevalence.

Even though the challenge methodology was similar across studies, Studies 2 and 3 had significant lower prevalence of SH in the samples collected when compared with Study 1. This limited to some extent the ability of differentiating treatment effects on SH prevalence. Consequently, none of the feed additive treatments was shown to significantly reduce SH prevalence and load when compared to the controls in both studies. Nevertheless, a general numerical trend of the feed additives in reducing SH numbers when compared to the controls was observed (Table 1). Experimental studies in our lab are designed to have a projected power of 80% to detect a 25% difference in protection as significant with 95% confidence. Therefore, we trust that the results presented herein are sound. However, one should not disregard that the smaller numerical differences between the treatments might be meaningful and cause great impact in a commercial production scenario. Reductions of 20% and 10% detection of *Salmonella* in cecae and boot swabs induced by BMD observed here can have a major influence on *Salmonella* detection downstream at the processing plant. Furthermore, the overall reduction of *Salmonella* load induced by BMD and zoalene can also be important when trying to alleviate *Salmonella* presence in the processing plant.

In conclusion, it was shown that NE enteritis episodes can result in *Salmonella* proliferation and that feed additive interventions that mitigate enteric disease can to some extent control *Salmonella* proliferation. Consequently, with the publication of stricter FSIS *Salmonella* compliance guidelines, broiler integrators should carefully evaluate the gut health control strategies in place in their production system.

REFERENCES

1. Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy 52:159-161. 2003.

2. Hofacre, C. L., G. F. Mathis, S. H. Miller, and M. W. LaVorgna. Use of Bacitracin and Roxarsone to Reduce Salmonella Heidelberg Shedding Following a Necrotic Enteritis Challenge Model1. The Journal of Applied Poultry Research 16:275-279. 2007.

3. Russell, S. M. The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors, and populations of Campylobacter spp. and Escherichia coli. Poultry Science 82:1326-1331. 2003.

4. Russell, S. M. A possible reason why more plants are failing the salmonella performance standard. Cooperative Extension Service, University of Georgia. 2005.

Table 1. Effects of various NE intervention strategies on *Salmonella* Heidelberg prevalence and load in broilers.

	% Positive							
	Spleen		Cecae		Boot Swabs		Carcass Rinse	
Study	#2	#3	#2	#3	#2	#3	#2	#3
Control	3.33	16.67	16.67	47.22	40.00	83.33	3.33	8.33
Probiotic	0.00	8.33	16.67	41.67	30.00	83.33	0.00	0.00
BMD	0.00	13.89	6.67	38.89	20.00	83.33	0.00	5.56
Zoamix	3.70	8.33	11.11	36.11	22.22	100.00	0.00	11.11

UNDERSTANDING RESISTANCE TO DIFFERENT INFECTIOUS BRONCHITIS VIRUS GENOTYPES

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ABSTRACT

In previous experiments, we have challenged major histocompatibility complex (MHC) congenic chicken lines available at UC Davis with an infectious bronchitis virus (IBV) M41 strain to assess resistance to IBV infection. We assessed clinical signs, tracheal thickness, viral load in tears and IgG and IgA levels in tears and sera and demonstrated that MHC haplotypes B2 and B19 were relatively the most resistant and susceptible respectively to an IBV challenge. The major differences between the two MHC haplotypes were found in viral load in tears at two dpi and in humoral responses at 14 dpi. In the present experiment, we used the resistant B2 and the susceptible B19 haplotype chickens to compare clinical, pathological and immunological differences between the two chicken lines after challenge with two different IBV genotypes, M41 and Arkansas Delmarva Poultry Industry (ArkDPI). We assessed respiratory signs, tracheal thickness, viral load in tears and IFN-β, IFN-γ, IgG and IgA levels in sera and tears. We also collected Harderian glands, CALT and tracheas at two and six dpi to perform RNA sequencing and investigate immune pathways that could be associated with the different levels of resistance in the two distinct MHC chicken lines. B2 haplotype chickens presented milder clinical signs, less tracheal inflammation and higher antibody production compared to B19 haplotype chickens in both viral challenges. These results support the evidence of B2 and B19 being resistant and susceptible respectively to IBV M41 and also to IBV ArkDPI. Cytokine assessments were unclear and further research is being performed to investigate the role of innate immune responses in resistance to IBV in these MHC congenic chicken lines.

INTRODUCTION

Infectious bronchitis (IB) is characterized by an upper respiratory, urogenital and reproductive illness in chickens. IBV is known for its genetic variability and phenotypic diversity. It is common to encounter new IBV genotypes able to successfully circumvent vaccination programs used in the poultry industry (10).

The chicken MHC is small and single dominantly expressed (8), showing strong association with protection or vulnerability to several infectious diseases (1, 4, 5), including IB (2, 3, 6). Understanding the mechanisms underlying resistance is essential to craft novel prevention strategies to IBV by stimulating components of the immune system that are not being stimulated by ordinarily used vaccines.

In previous experiments, we have demonstrated that MHC haplotype B2 chickens were relatively resistant and that MHC haplotype B19 chickens were relatively susceptible to our challenge with IBV Massachusetts 41 (M41). The major differences between the two MHC haplotypes were found in viral load in tears at 2 days post-infection (dpi) and in humoral responses at 14 dpi (6). In the present experiment, our goal was to evaluate differences in clinical signs, tracheal thickness, viral load, cytokine production and humoral responses between relatively resistant and susceptible MHC congenic chicken lines after challenges with two IBV genotypes, M41 and ArkDPI. We are currently analyzing the tracheal transcriptome to investigate immune pathways elicited in B2 and B19 haplotype chickens and the possible differences in viral microbiome composition.

MATERIALS AND METHODS

The relatively resistant 331/B2 and susceptible 335/B19 chicken lines were used in this experiment (6). A total of 150 chickens – 75 of each line – were divided into six groups with 25 birds each. The challenge was performed at 23 days of age with a $5x10^7$ median embryo infectious dose (EID_{50}) of either IBV M41 or IBV ArkDPI in a final volume of 200 µL via oculonasal route. Control groups were unchallenged.

At two and six dpi, respiratory signs and viral load in tears were assessed in all birds as described previously (6). Eight birds per group were euthanized at two and six dpi and tracheas were collected to

perform histomorphometrical analysis. In addition, sera and tears were collected to detect IFN-β and IFN-γ by enzyme-linked immunosorbent assay (ELISA). Harderian glands, tracheas and conjunctiva-associated lymphoid tissues (CALT) were collected to perform RNA-sequencing to explore immunological pathways that might be associated with susceptible and resistant chicken lines and to investigate their upper respiratory tract virome. At 14 dpi, sera and tears were collected from the remaining birds to assess IFN-β, IFN-γ, anti-IBV IgG and IgA levels by ELISA.

Clinical signs, viral load, histomorphometry measurements, cytokine concentrations and antibody titers were analyzed individually and compared by one-way ANOVA followed by Tukey multiple comparisons test using GraphPad Prism software (GraphPad, La Jolla CA, USA). Statistical differences were considered at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

At two dpi, there were no differences in respiratory sign indices or tracheal epithelial thicknesses between the challenged groups. At six dpi, the resistant B2 haplotype chickens presented less severe respiratory signs and less tracheal inflammation than the susceptible B19 chickens, regardless of the IBV genotype (Figure 1). B2 haplotype chickens challenged with IBV ArkDPI presented the lowest viral load at two dpi. No differences between the challenged groups were detected at six dpi.

It was not possible to draw conclusions about innate immune responses through our IFN-β and IFN-γ assessments in sera and tears. Some possible explanations are: (1) Inappropriate sample collection dates to assess innate immune responses; (2) Lack of environmental control which might have led to innate immunity stimulation before the IBV challenge in all groups. This situation might have masked patterns or differences between the groups. Further in vivo experiments are being performed in battery cages looking for a better control of the environment.

The major differences between the two chicken lines, even though non-significant, were observed in humoral immune responses. B2 haplotype chickens presented higher IBV-specific IgG response in sera compared to B19 chickens at 14 dpi (Figure 2A). In order to assess local humoral responses, we assessed IgG and IgA levels in tears. IgG levels in tears were statistically higher in B2 than in B19 haplotype chickens in both viral challenges ($p<0.05$, Figure 2B). The high titers of local IgG is most likely due to the transport of IgG from sera and to the production of IgG by plasma cells in the Harderian glands and CALT (9). IgA results in tears were also higher in B2 haplotype chickens than in B19, especially in birds challenged with IBV M41 ($p<0.05$, Figure 2C). These results are of paramount importance since generation of IBV neutralizing antibodies in the primary infection site rather than systemic response alone should be the goal of IBV vaccines.

Harderian glands, CALT and tracheas collected at two and six dpi are still being processed in order to assess transcriptomic changes induced by both challenges (IBV M41 and ArkDPI). We will also investigate changes in the tracheal virome from both chicken lines.

The results from this experiment suggest that 331/B2 is relatively resistant to both IBV M41 and ArkDPI, validating the B2/B19 resistant-susceptible model as described by us (6) and others (3, 5, 7). Further research will be focused on investigating innate immune responses of these lines in more depth.

(The full-length article will be submitted for publication in Avian Diseases journal.)

REFERENCES

1. Abplanalp, H., K.A. Schat, and B.W. Calnek. Resistance to Marek's disease of congenic lines differing in major histocompatibility haplotypes to 3 virus strains. In: International Symposium on Marek's Disease. B.W. Calnek and J.L. Spencer. American Association of Avian Pathologists, Ithaca, NY. pp 347-358. 1984.

2. Bacon, L.D., D.B. Hunter, H.M. Zhang, K. Brand, and R. Etches. Retrospective evidence that the MHC (B haplotype) of chickens influences genetic resistance to attenuated infectious bronchitis vaccine strains in chickens. Avian Pathol 33:605-609. 2004.

3. Banat, G.R., S. Tkalcic, J.A. Dzielawa, M.W. Jackwood, M.D. Saggese, L. Yates, R. Kopulos, W.E. Briles, and E.W. Collisson. Association of the chicken MHC B haplotypes with resistance to avian coronavirus. Dev Comp Immunol 39:430-437. 2013.

4. Briles, W.E., R.W. Briles, D.L. Pollock, and M. Pattison. Marek's disease resistance of B (MHC) heterozygotes in a cross of purebred Leghorn lines. Poult Sci 61:205-211. 1982.

5. Collisson, E., L. Griggs, and Y. Drechsler. Macrophages from disease resistant B2 haplotype chickens activate T lymphocytes more effectively than macrophages from disease susceptible B19 birds. Dev Comp Immunol 67:249-256. 2017.

6. da Silva, A.P., R. Hauck, H. Zhou, and R.A. Gallardo. Understanding immune resistance to infectious bronchitis using major histocompatibility complex chicken lines. Avian Dis 61:358-365. 2017.

7. Dawes, M.E., L.M. Griggs, E.W. Collisson, W.E. Briles, and Y. Drechsler. Dramatic differences in the response of macrophages from B2 and B19 MHC-defined haplotypes to interferon gamma and

polyinosinic:polycytidylic acid stimulation. Poult Sci 93:830-838. 2014.

8. Kaufman, J. The Avian MHC. In: Avian Immunology, 2 ed. K.A. Schat, B. Kaspers and P. Kaiser. Academic Press, Boston. pp 149-167. 2014.

9. Toro, H., P. Lavaud, P. Vallejos, and A. Ferreira. Transfer of IgG from serum to lachrymal fluid in chickens. Avian Dis 37:60-66. 1993.

10. Toro, H., V.L. van Santen, and M.W. Jackwood. Genetic diversity and selection regulates evolution of infectious bronchitis virus. Avian Dis 56:449-455. 2012.

Figure 1. Respiratory signs index and tracheal epithelium thickness of MHC congenic chicken lines 331/B2 and 335/B19 unchallenged (controls) and challenged with IBV M41 or ArkDPI. **(A)** Respiratory signs at 6 dpi. **(B)** Tracheal epithelial thickness at 6 dpi. Superscripts indicate significant differences ($p<0.05$).

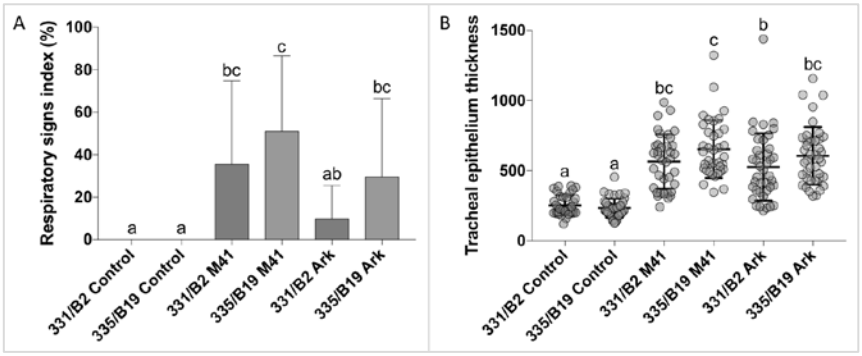
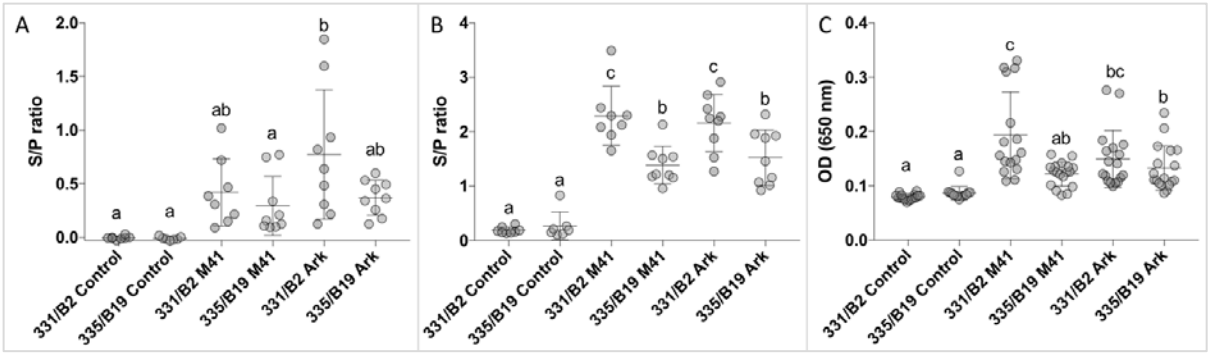


Figure 2. Humoral responses of MHC B congenic chicken lines 331/B2 and 335/B19 unchallenged and challenged with IBV M41 or ArkDPI. **(A)** IgG levels in sera at 14 dpi. **(B)** IgG levels in tears at 14 dpi. **(C)** IgA levels in tears at 14 dpi. Superscripts indicate significant differences ($p<0.05$).



EFFICACY OF A DUAL-INSERT VECTORED VACCINE AGAINST MAREK'S DISEASE, NEWCASTLE DISEASE, AND BURSAL DISEASE

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SUMMARY

Vectored vaccines using the turkey herpesvirus (HVT) as a backbone to express antigens of Newcastle disease virus (NDV) or infectious bursal disease virus (IBDV) are efficacious and widely used. These vaccines are administered in the hatchery to chicken embryos (*in ovo*) or to day-of-age chickens (subcutaneous). Due to concerns related to replication interference of the vaccine vectors, rHVT/NDV and rHVT/IBDV vaccines are not administered concurrently, leaving to the producer the decision of which vectored vaccine to use. In order to overcome vector replication interference and allow for concomitant vaccination against Newcastle disease (ND) and infectious bursal disease (IBD) with a single vectored vaccine, a novel dual-insert, HVT-vectored vaccine expressing antigens of NDV and IBDV has been developed. This dual-insert vaccine is stable, safe and efficacious. After passage of the vaccine in chickens and chicken embryo fibroblasts, both inserted genes were stably expressed and the vaccine did not become pathogenic. Efficacy of this dual-insert, HVT-vectored vaccine in combination with the Marek's disease virus serotype 2 strain SB-1 was demonstrated against very virulent Marek's disease (MD) in specific pathogen free (SPF) chickens, and against ND and IBD in SPF and maternal antibody positive chickens. The stability, safety and efficacy of the novel dual-insert vaccine position it as a pivotal tool toward controlling MD, ND and IBD in commercial poultry operations.

INTRODUCTION

Control of infectious diseases is one of the most important concerns in the poultry industry. In particular, ND, MD, and IBD are widespread and have caused significant economic losses worldwide because of their high transmission and mortality rates. Many vaccines have been developed and investigated to control these diseases, including classical and recombinant vaccines (1-3, 5, 7, 10). Several recombinant vaccines using HVT as a vector to express immunogenic genes from NDV or IBDV are commercially available (3, 5). The HVT used in these recombinants is an alpha-herpesvirus isolated from turkeys and it is antigenically related to Marek's disease virus (MDV) (6). HVT has been used as a vaccine against MD in chickens for decades either alone or in combination with other Marek's disease strains, such as SB-1 (serotype 2 MDV) and Rispens CVI 988 (serotype 1 MDV), depending on the virulence of the MDV field challenge (1, 10-12). While Rispens and SB-1 synergize with HVT for the efficacy against MD and do not interfere with the efficacy of the rHVT vaccines (5, 13-15), interference with the efficacy of inserted genes occurs when rHVT vaccines are simultaneously administered with a conventional HVT vaccine (13).

In order to overcome vector replication interference and allow for concomitant vaccination against MD, ND, and IBD with a single dose of vaccine at hatchery, a novel dual-insert, HVT-vectored vaccine expressing antigens of NDV and IBDV (rHVT/ND/IBD) has been developed. This dual-insert vaccine carries the virion protein 2 (VP2) gene of IBDV and the fusion protein (F) gene of NDV. The objective of the present study was to evaluate genetic stability and safety of this vaccine, and efficacy of it against MD, ND, and IBD in SPF and maternal antibody positive chickens.

MATERIALS AND METHODS

Genetic stability and safety of HVT/ND/IBD. Expression of the NDV F protein and IBDV VP2 protein from the recombinant dual-insert, HVT-vectored vaccine was evaluated by western blot analysis and an immunofluorecence assay (IFA). Briefly, for western blot analysis, lysates of chicken embryo fibroblasts (CEF) infected with rHVT/ND/IBD master seed virus (MSV) were separated on 7.5% acrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membranes. Rabbit anti-NDV F serum was used to detect NDV F protein and monoclonal antibody R63 was used to detect IBDV VP2 protein. For the IFA, monolayers of CEF infected with rHVT/ND/IBD were fixed with a mixture of methanol and acetone (volume ratio = 1:2). The fixed monolayer was incubated with a combination of primary monoclonal antibodies anti-

NDV F (3-1G5/4E, isotype IgG1) and anti-IBDV VP2 (R63, isotype IgG2a), and then with a combination of the secondary antibodies PE anti-mouse IgG1 and FITC anti-mouse IgG2a. Stained viral plaques were observed under an inverted fluorescence microscope.

A backpassage study and an overdose study were conducted to investigate genetic stability and safety of rHVT/ND/IBD. For the backpassage, virus was passed five times in SPF chickens. Chickens from the fifth passage were observed for clinical signs of MD for 45 days. After 45 days, chickens were necropsied and observed for grossly observable MD lesions. Also at the fifth passage, virus isolation was attempted. Recovered virus was further propagated and used for various assays to confirm genetic and phenotypic stability of rHVT/ND/IBD. For the overdose study, rHVT/ND/IBD at 45,000 pfu/chicken was inoculated either by the in ovo route into 18-day-old SPF embryos or subcutaneously (SQ) into one-day-old SPF chicks. Chickens were observed until 18 weeks of age for any adverse vaccine reactions and for any clinical signs associated with MD. At 18 weeks of age, chickens were necropsied and observed for grossly observable MD lesions.

Efficacy against very virulent RB1/B MDV challenge in SPF chickens. SPF chicks were divided into five treatment groups. One group of embryos at 18 days of incubation was vaccinated via the in ovo route with rHVT/ND/IBD and SB-1. One group of chicks at one day of age was vaccinated via the SQ route with rHVT/ND/IBD and SB-1. A third group was vaccinated SQ at one day of age with a commercial Marek's Disease serotype 3 vaccine. A fourth group was placebo-vaccinated by the in ovo route and served as a positive challenge control, and a fifth group was placebo-vaccinated SQ and served as a negative control. At five days of age, chickens were challenged with the very virulent RB1/B MDV strain. After challenge, chickens were observed until seven weeks of age, necropsied and observed for MD lesions.

Efficacy of NDV and IBDV challenges in commercial layers. Maternal antibody positive commercial layer chickens were used in this study. For Trial 1, rHVT/ND/IBD and SB-1 were administered via the in ovo route to embryos at 18 days of incubation or the SQ route to chicks at one day of age. Placebo-vaccinated embryos served as an in ovo positive challenge control. Chickens were challenged at eight weeks of age with 104.0 50% embryo infective dose (EID50) of Newcastle Disease Texas GB strain. After NDV challenge, chickens were observed for clinical signs of ND. Analogous treatment groups were used in Trial 2, and chickens were challenged at 10 weeks of age with 10 4.0 EID50 of the USDA IBDV STC NVSL strain. After IBDV challenge, chickens were observed for clinical signs of IBD. Four days after challenge, all chickens were necropsied to observe gross lesions of IBD.

RESULTS

Genetic stability of HVT/ND/IBD. Genetic stability of rHVT/ND/IBD was shown by Western blot analysis. The NDV F protein (~60 KD) was detected in CEF infected with rHVT/ND/IBD MSV, rHVT/ND/IBD MSV+7 and rHVT/ND/IBD isolated from the fifth passage in chickens (Figure 1A) and not in noninfected CEF or CEF infected with the backbone HVT. Likewise, IBDV VP2 (~37 KD) was also specifically detected in the same CEF monolayers (Figure 1B). The genetic stability of rHVT/ND/IBD MSV+7 was further characterized by dual-staining IFA. Expression of NDV F protein and IBDV VP-2 protein were detected in the same viral plaques (Figure 1C). Microscopic inspection of viral plaques established that every viral plaque expressed both viral protein inserts (data not shown).

Safety of rHVT/ND/IBD. Safety of rHVT/ND/IBD was shown in a backpassage study and an overdose study. After five consecutive passages of rHVT/ND/IBD in chickens, no clinical signs associated with MD or grossly observable MD lesions were observed, confirming that the vaccine virus did not become pathogenic (Table 1). As expected, rHVT/ND/IBD was isolated from chickens after five passages, confirming the presence of the vaccine virus (Table 1). Chickens from the overdose study showed no adverse vaccine reactions or any clinical signs associated with MD, and no grossly observable MD lesions after necropsy at 18 weeks post vaccination (Table 2). Furthermore, in ovo administration of an overdose of rHVT/ND/IBD did not affect hatchability. In the placebo-vaccinated, RB1/B-challenged group, most of the chickens developed grossly observable lesions of RB1/B, confirming that the chickens used in this study were susceptible to MD (Table 2).

Efficacy against very virulent RB1/B MDV challenge in SPF chickens. rHVT/ND/IBD in combination with SB-1 was efficacious against challenge by the very virulent RB1/B MDV. Forty out of forty-five (89%) chickens vaccinated by the in ovo route with rHVT/ND/IBD and SB-1, and 38/45 (84%) of those vaccinated SQ, showed no gross lesions of MD and were protected against RB1/B MDV challenge (Figure 2A). Less than 80% of chickens vaccinated with a commercial Marek's Disease serotype 3 vaccine were protected from RB1/B MDV challenge, demonstrating a valid challenge.

Efficacy of NDV and IBDV challenges in commercial layers. rHVT/ND/IBD was efficacious against challenge by NDV Texas GB strain and

against challenge by IBDV STC strain in maternal antibody positive commercial layer chickens (Figure 2B) and in SPF chickens (data not shown). Thirty out of thirty (100%) chickens vaccinated by the in ovo route with rHVT/ND/IBD, and 30/30 (100%) vaccinated SQ, showed no gross lesions of IBD and were protected against IBDV STC challenge (Figure 2B). Thirty out of thirty (100%) chickens vaccinated by the in ovo route with rHVT/ND/IBD, and 30/30 (100%) vaccinated SQ, showed no clinical signs of ND and were protected against NDV Texas GB challenge (Figure 2B).

DISCUSSION

This study demonstrated the genetic stability, safety and efficacy of a dual-insert rHVT vaccine expressing antigens of NDV and IBDV, i.e. rHVT/ND/IBD. Genetic stability was demonstrated after passages of the vaccine in cell culture, as well as chickens. A dual IFA on infected monolayers determined that every rHVT/ND/IBD plaque expressed the inserted NDV F gene and IBDV VP2 gene, and Western blot analyses confirmed that the expressed inserts were full length. Therefore, the insertion of two exogenous antigens in the HVT vector did not compromise the stability of either insert that exists in the rHVT vaccines expressing a single insert (3, 4, 9).

Safety of rHVT/ND/IBD was demonstrated by an overdose study and a backpassage study. In the overdose study, rHVT/ND/IBD inoculated either by the in ovo route to SPF embryos at 18 days of incubation or SQ into day-of-age SPF chicks did not cause any adverse vaccine reactions or lesions. In the backpassage study, rHVT/ND/IBD did not become pathogenic after five passages in chickens. These results demonstrate that the dual insertion did not change the safety profile of the HVT, which has been used extensively as a vaccine against MD for decades without causing any problems in the field (1, 8).

The rHVT/ND/IBD vaccine in combination with Marek's disease serotype 2 (SB-1) administered via the in ovo or SQ route was highly efficacious against MD, ND, and IBD in both SPF chickens and commercial layers. Therefore, concomitant expression of the two viral exogenous genes by the same HVT vector did not compromise the protection induced by NDV F or IBDV VP2 genes expressed individually in the existing rHVT/ND and rHVT/IBD vaccines. A tremendous advantage of this dual-insert vaccine is that it allows for concomitant vaccination against MD, ND and IBD with a single vaccine dose in the hatchery. Even though there are several commercially available highly efficacious rHVT vaccines against ND and IBD (2, 3), these vaccines are not administered concurrently due to concerns that replication interference between vaccine vectors can compromise the immune response against the inserted genes (13). As expected, the combination between HVT and SB-1 synergized to confer protection against very virulent Marek's disease virus RB1/B strain and the SB-1 fraction did not interfere with the protection induced by rHVT/ND/IBD against ND and IBD. In conclusion, rHVT/ND/IBD is a promising tool towards the control of these three widespread and detrimental diseases for the poultry industry.

(The full-length article will be submitted to *Vaccine*.)

REFERENCES

1. Burmester, B. R., H. G. Purchase, and W. Okazaki. Long-term experiences with the herpesvirus of turkeys (HVT) as a vaccine against Marek's disease. Prog Immunobiol Stand 5:132-138. 1971.

2. Darteil, R., M. Bublot, E. Laplace, J. F. Bouquet, J. C. Audonnet, and M. Riviere. Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. Virology 211:481-490. 1995.

3. Esaki, M., A. Godoy, J. K. Rosenberger, S. C. Rosenberger, Y. Gardin, A. Yasuda, and K. M. Dorsey. Protection and antibody response caused by turkey herpesvirus vector Newcastle disease vaccine. Avian Dis 57:750-755. 2013.

4. Esaki, M., L. Noland, T. Eddins, A. Godoy, S. Saeki, S. Saitoh, A. Yasuda, and K. M. Dorsey. Safety and efficacy of a turkey herpesvirus vector laryngotracheitis vaccine for chickens. Avian Dis 57:192-198. 2013.

5. Ishihara, Y., M. Esaki, S. Saitoh, and A. Yasuda. Combination of Two Marek's Disease Virus Vectors Shows Effective Vaccination Against Marek's Disease, Infectious Bursal Disease, and Newcastle Disease. Avian Dis 60:473-479. 2016.

6. Kawamura, H., D. J. King, Jr., and D. P. Anderson. A herpesvirus isolated from kidney cell culture of normal turkeys. Avian Dis 13:853-863. 1969.

7. Morgan, R. W., J. Gelb, Jr., C. R. Pope, and P. J. Sondermeijer. Efficacy in chickens of a herpesvirus of turkeys recombinant vaccine containing the fusion gene of Newcastle disease virus: onset of protection and effect of maternal antibodies. Avian Dis 37:1032-1040. 1993.

8. Okazaki, W., H. G. Purchase, and B. R. Burmester. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Dis 14:413-429. 1970.

9. Perozo, F., A. P. Villegas, R. Fernandez, J. Cruz, and N. Pritchard. Efficacy of single dose recombinant herpesvirus of turkey infectious bursal disease virus (IBDV) vaccination against a variant IBDV strain. Avian Dis 53:624-628. 2009.

10. Purchase, H. G., W. Okazaki, and B. R. Burmester. Field trials with the herpes virus of turkeys (HVT) strain FC126 as a vaccine against Marek's disease. Poult Sci 50:775-783. 1971.

11. Rispens, B. H., H. van Vloten, N. Mastenbroek, J. L. Maas, and K. A. Schat. Control of Marek's disease in the Netherlands. II. Field trials on vaccination with an avirulent strain (CVI 988) of Marek's disease virus. Avian Dis 16:126-138. 1972.

12. Schat, K. A., and B. W. Calnek. Characterization of an apparently nononcogenic Marek's disease virus. J Natl Cancer Inst 60:1075-1082. 1978.

13. Slacum, G. The compatibility of HVT recombinants with other Marek's disease vaccines. In: 58th Western Poultry Disease Conference. Sacramento, CA. p 285. 2009.

14. Witter, R. L., and L. F. Lee. Polyvalent Marek's disease vaccines: safety, efficacy and protective synergism in chickens with maternal antibodies. Avian Pathol 13:75-92. 1984.

15. Witter, R. L., L. F. Lee, and A. M. Fadly. Characteristics of CVI988/Rispens and R2/23, two prototype vaccine strains of serotype 1 Marek's disease virus. Avian Dis 39:269-284. 1995.

Figure 1. A and B. Western blot analysis. A. Use of rabbit anti-NDV F serum produced the expected ~60-kD band in CEF infected with rHVT/ND/IBD MSV, MSV+7 and backpassage (BP+5). B. Use of anti-IBDV VP2 monoclonal antibody R63 produced the expected 36-kD band for rHVT/ND/IBD MSV, rHVT/ND/IBD MSV+7 and BP+5. C. Dual staining IFA. A mix with the primary antibodies anti-NDV F protein (3-1G5) and anti-IBDV R63 and secondary antibodies PE anti-IgG1 and FITC anti-IgG2a stained F protein and VP-2 protein in the same rHVT/IBD plaques.

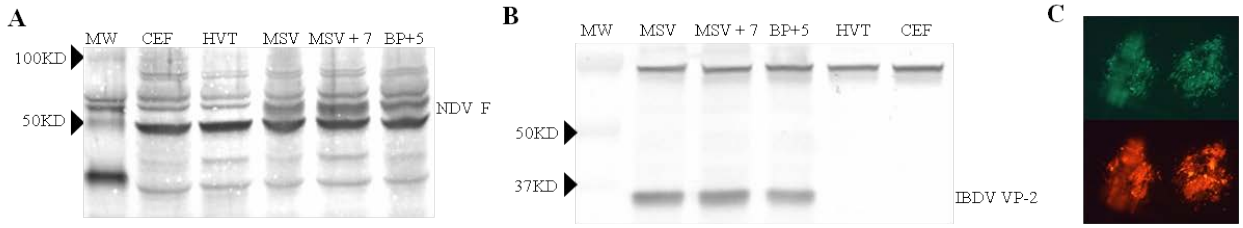


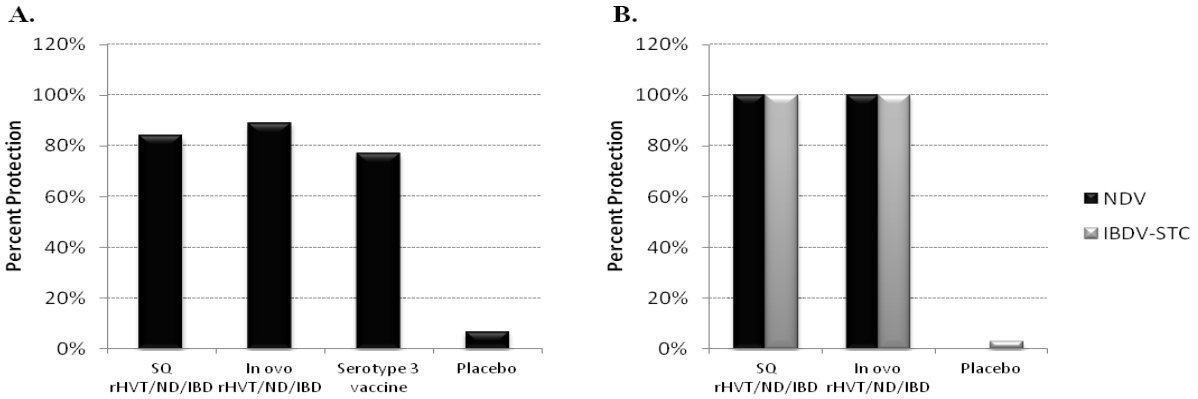
Table 1. Safety of rHVT/ND/IBD in a backpassage study results from the fifth passage of rHVT/ND/IBD in chickens.

Treatment	No. Chickens Placed	MD Clinical Signs	Necropsy Results	Virus Isolation
Vaccinates	25	Negative	Negative	Positive
Controls	10	Negative	Negative	Negative

Table 2. Safety of rHVT/ND/IBD in an overdose study.

Treatment	No. Hatched/ No. Embryos Set (% Hatchability)	No. Chicks Placed (No. Surviving > 5 days)	Mortality (No. with Gross Lesions)	No. Chickens with Adverse Reactions or Clinical Signs of MD	No. Chickens at Necropsy (No. with Gross Lesions)
rHVT/ND/IBD in ovo-vaccinated	58/78 (74%)	50 (50)	8 (0)	0	42 (0)
rHVT/ND/IBD SQ-vaccinated	51/78 (65%)	50 (50)	2 (0)	0	48 (0)
placebo SQ-vaccinated, RB1/B-challenged (positive control)	60/78 (77%)	50 (50)	39 (36)	Not applicable	11 (11)
placebo *in ovo*-vaccinated, non-challenged (negative control)	56/78 (72%)	50 (49)	3 (0)	0	46 (0)

Figure 2. Efficacy of rHVT/ND/IBD. **A.** Marek's RB1/B challenge. **B.** NDV and IBDV challenge.



IN OVO DELIVERED CPG OLIGONUCLEOTIDES (ODNS) PROTECT CHICKENS AGAINST INFECTIOUS BRONCHITIS: POTENTIAL ROLES OF INNATE AND ADAPTIVE IMMUNE CELLS

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SUMMARY

In ovo delivered CpG ODNs protect chickens encountered with bacterial and viral infections after hatch. Although, *in ovo* delivered CpG ODNs have been shown to reduce infectious bronchitis virus (IBV) loads in the respiratory tract pre-hatch, whether *in ovo* delivered CpG ODNs are capable of protecting chickens post-hatch is unknown. In light of these evidence, we sought to find out the protective effects of *in ovo* delivered CpG ODNs against IBV infection in post-hatch chickens and its correlation with the immune cell recruitment. We found that treatment of embryo day (ED) 18 eggs with CpG ODNs and succeeding challenge at day 1 post-hatch with IBV Massachusetts-type 41 (M41), provided significant protection against IBV in birds when compared to controls that received control ODNs. Correlating with this protection, we found increased recruitments of macrophages as well as B cells and T cell sub sets in the respiratory tract of chickens in response to *in ovo* CpG ODN delivery.

In conclusion, it may be inferred that pre-hatch delivery of CpG ODNs leads to increased recruitment of innate and adaptive immune cells as such, could protect chickens from IBV infection encountered post -hatch.

INTRODUCTION

Toll-like receptors (TLRs) are a family of one of many germ line encoded pattern recognizing receptors (PRRs) located on the surface or within endosomal compartments of cells (9). These receptors are crucial in recognizing whole or segments of microbial pathogens and mounts key host immune defenses against inciting agents. Among the well-recognized TLRs, TLR9 (in mammals) / TLR 21 (in birds) are the only receptors capable of distinguishing bacterial and viral DNA containing unmethylated CpG motifs (10). Several studies have demonstrated this immune-stimulatory activity of synthetic CpG ODNs in murine, chicken, neonatal piglets and nonhuman primates (2, 3, 5, 8, 15, 16). The protection provided by CpG ODNs against lethal challenges of intracellular pathogens such as *Mycobacterium bovis* (6), *Listeriamonocytogenes* (13) *and Leishmania major* (17) in mice are well documented.

While CpG ODNs are known to induce a production of an array of cytokines, chemokines and pro inflammatory molecules that are believed to play an integral role in protecting the host against disease-inciting pathogen (4, 5, 11, 12), it is also reported that CpG ODNs are capable of stimulating innate immune cell recruitment such as dendritic cells, macrophages, B cells (1) and even T lymphocytes (co-stimulatory activity seen when applied together with a T cell receptor signal) (7). In chickens, when CpG DNA was applied *in ovo* as a prophylactic agent, significant increases in recruitment of macrophages (14), B cells, CD4+ and CD8+ T cells post-hatch was observed with a parallel protective response against ILTV infection in terms of reduced virus replication in the respiratory system (14) and in minimizing shedding of the virus.

In line with these findings, we hypothesized that *in ovo* delivery of CpG pre-hatch may induce antiviral responses that provides protection of post-hatch chickens against IBV infection through reduced morbidity and mortality correlating with increased recruitment of innate and adaptive immune cells.

MATERIALS AND METHODS

For survival evaluation of IBV infection, ED 18 SPF eggs were delivered with CpG ODNs *in ovo* via chorioallantoic route and another portion with control ODNs via the same route. On the day of hatch, birds were intra-tracheally challenged with 2.75×10^4 EID_{50} of IBV M41 strain and monitored for clinical signs on a daily basis. In addition, trachea and lungs were harvested from 3 dpi birds in RNA-Save (Biological Industries, Frogga Bio, Toronto ON, Canada) for virus

detection by PCR array analysis, in optimum cutting temperature (OCT) compound for indirect immunofluorescent assay to quantify macrophage, B cell and T cell subsets and in 10% formalin for histopathological examination.

Trizol reagent (Ambion, Invitrogen Canada Inc., Burlington, ON, Canada) was used to extract the viral RNA from lung tissue homogenates and two step RT-PCR was performed for IBV N gene detection.

For immune cell staining, unlabeled monoclonal antibody specific for chicken macrophages, KUL01 (Southern Biotech, Birmingham, Alabama, USA), CD4 (Southern Biotech, Birmingham, Alabama, USA),CD8α(Southern Biotech, Birmingham, Alabama, USA) and IgM (Southern Biotech, Birmingham, Alabama, USA) were used as primary antibodies. For macrophages, IgM and CD8 cells Dylight® 550 conjugated goat anti-mouse IgG (H+L) (Bethyl Laboratories Inc., Montgomery, TX, USA) was used as secondary antibody and for CD4 cells biotinylated goat anti-mouse IgG (H+L) (Southern Biotech, Birmingham, Alabama, USA) followed by Dylight® 488 was used. The fluorescence signal was imaged using an epifluorescence microscope after adding Vectashield mounting medium containing 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) nuclear stain (Vector Laboratories Inc., Burlingame, CA, USA). It was then quantified using image J software (National Institute of Health, Bethesda, Maryland, USA).

Formalin fixed tissues were sent to the Histopathology Diagnostic Services Unit at the University of Calgary Faculty of Veterinary Medicine for sectioning and staining with hematoxylin and eosin (H&E). The histological changes were imaged under a light microscope.

RESULTS

We found that TLR-21 ligand CpG ODNs, when delivered to ED 18 embryos *in ovo*, significantly improve the IBV induced mortality, morbidity and histopathology with associated increased recruitment of immune cells such as macrophages, T and B cells.

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

REFERENCES

1. Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM (2011) CpG DNA as a vaccine adjuvant. Expert review of vaccines 10:499-511.

2. Cai H, Kuang Z, Huang K, Shi J, Zhao X, Chu P, Huang C, Ming F, Xia F, Yang J (2014) CpG oligodeoxynucleotide protect neonatal piglets from challenge with the enterotoxigenic E. coli. Veterinary immunology and immunopathology 161:66-76.

3. Cowdery JS, Chace JH, Yi A-K, Krieg AM (1996) Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. The Journal of Immunology 156:4570-4575.

4. Dar A, Potter A, Tikoo S, Gerdts V, Lai K, Babiuk LA, Mutwiri G (2009) CpG oligodeoxynucleotides activate innate immune response that suppresses infectious bronchitis virus replication in chicken embryos. Avian diseases 53:261-267.

5. Dar A, Tikoo S, Potter A, Babiuk LA, Townsend H, Gerdts V, Mutwiri G (2014) CpG-ODNs induced changes in cytokine/chemokines genes expression associated with suppression of infectious bronchitis virus replication in chicken lungs. Veterinary immunology and immunopathology 160:209-217.

6. Freidag BL, Melton GB, Collins F, Klinman DM, Cheever A, Stobie L, Suen W, Seder RA (2000) CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of Mycobacterium bovis BCG vaccination in mice challenged with M. tuberculosis. Infection and immunity 68:2948-2953.

7. Häcker G, Redecke V, Häcker H (2002) Activation of the immune system by bacterial CpG-DNA. Immunology 105:245-251.

8. Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, Suparto I, Rasmussen WL, Waldschmidt M, Sajuthi D, Purcell RH (2000) Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. The Journal of Immunology 164:1617-1624.

9. He H, MacKinnon KM, Genovese KJ, Kogut MH (2011) CpG oligodeoxynucleotide and double-stranded RNA synergize to enhance nitric oxide production and mRNA expression of inducible nitric oxide synthase, pro-inflammatory cytokines and chemokines in chicken monocytes. Innate immunity 17:137-144.

10. Hemmi H, Akira S (2002) A novel Toll-Like receptor that recognizes bacterial DNA. Microbial DNA and Host Immunity:39-47.

11. Kameka AM, Haddadi S, Kim DS, Cork SC, Abdul-Careem MF (2014) Induction of innate immune response following infectious bronchitis corona virus infection in the respiratory tract of chickens. Virology 450:114-121.

12. Kato A, Homma T, Batchelor J, Hashimoto N, Imai S, Wakiguchi H, Saito H, Matsumoto K (2003) Interferon-α/β receptor-mediated selective induction of a gene cluster by CpG oligodeoxynucleotide 2006. BMC immunology 4:8.

13. Krieg AM, Love-Homan L, Yi A-K, Harty JT (1998) CpG DNA induces sustained IL-12 expression in vivo and resistance to Listeria monocytogenes challenge. The Journal of Immunology 161:2428-2434.

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

15. Yamaguchi Y, Harker JA, Wang B, Openshaw PJ, Tregoning JS, Culley FJ (2012) Preexposure to CpG protects against the delayed effects of neonatal respiratory syncytial virus infection. Journal of virology 86:10456-10461.

16. Yi A, Klinman D, Martin T, Matson S, Krieg A (1996) Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. Journal of immunology (Baltimore, Md: 1950) 157:5394.

17. Zimmermann S, Egeter O, Hausmann S, Lipford GB, Röcken M, Wagner H, Heeg K (1998) Cutting edge: CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. The Journal of Immunology 160:3627-3630.

BACKYARD POULTRY FLOCKS *SALMONELLA* SPP. SEROPREVALENCE

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INTRODUCTION

As backyard poultry grows as a hobby in the United States there is an increased worry that poultry will become infected with *Salmonella* spp. and transfer this infection to those commingling with birds or consuming their products. Within the next five years 4% of the population of some of the largest cities in the United States plan on getting chickens. Currently, almost 1% of the population in the same cities already own chickens (NARMS-USDA). Backyard poultry owners lack reliable sources of information to care for the birds and keep them in good health (8). The common source of information for backyard flock proprietors are non-academic websites which translates in to substandard biosecurity, health, and management practices (1, 4). In this study we focused on paratyphoid *Salmonella* and *Salmonella* Pullorum causing food safety issues in humans and chick septicemic disease with high mortality, respectively. A countrywide *Salmonella* outbreak in humans, linked to live poultry in backyard flocks has been ongoing during 2017 with 1120 human cases (2)

The purpose of this study was to determine correlation between backyard flock biosecurity and management, and *Salmonella* prevalence in small flocks. These results will help direct biosecurity measures needed to be taken by backyard producers in order to diminish the risk of *Salmonella* outbreaks and spread, and to preserve human-animal health.

MATERIALS AND METHODS

Biosecurity survey. A survey was created to assess the management and biosecurity of backyard chicken flocks. The survey was modeled after a biosecurity assessment of commercial poultry facilities (7). The survey consists of 34 questions and was conducted face-to-face with the participants at their property (5). The same interviewer was used every time the survey was conducted.

Backyard flocks. All animal experimental procedures were approved by the University of California- Davis Institute Animal Care and Use Committee. Backyard poultry flocks were located and contacted using the California Backyard Poultry Census (http://ucanr.edu/sites/poultry/California_Poultry_Census/). The size of the flocks ranged from 2-400 birds and all flocks were located in Yolo, Sonoma, Napa, Alameda, and Yuba counties in California. All the flocks were visited between January and September, 2016.

Serological tests. Antibodies against paratyphoid *Salmonella* were tested using a commercially available ELISA kit targeting a *Salmonella* flagellar antigen. Antibodies against *Salmonella* Pullorum were tested using a plate agglutination assay. Statistical analysis was performed using chi-square test, statistical significance was determined at $p<0.05$.

RESULTS

Forty-one backyard flocks with a total of 554 birds were sampled. Flocks contained a mix of breeds, varied from two to 400 birds, ages ranged from two to 10 years old. All flock owners (41/41) answered the questionnaire when sampling occurred. Paratyphoid *Salmonella* was detected by the ELISA kit in five out of 41 unique flocks and was suspect in 13, with an overall flock prevalence of 20% (8/41). Out of the 540 samples tested by plate agglutination tests, 77 were positive for *S.* Pullorum (14.3%). If we consider the flocks 33/41 had positive birds (80.5%).

Flock-based categorized *Salmonella* positivity showed no significance; however, individual bird categorized data showed statistically significant trends when survey questions were correlated with *Salmonella* positivity. Lack of use of dedicated poultry clothing and footwear showed significance increase in both paratyphoid *Salmonella* ($p<0.05$) and *S.* Pullorum ($p<0.05$). The presence of wild birds near flocks

showed a significance increase for *S*. Pullorum ($p<0.05$). Presence of rodents showed significance increase of *S*. Pullorum ($p<0.05$). Obtaining birds from NPIP hatchery showed a significant decrease in *S*. Enteritidis ($p<0.05$).

DISCUSSION

Backyard poultry owners showed to be lackadaisical when it comes to implementing simple biosecurity measures such as, using dedicated shoes or clothes to work with poultry. It has been previously shown that diseases can stay in boot crevices even after boot disinfection. (7). This habit correlates with high paratyphoid *Salmonella* and *S*. Pullorum prevalence. Most of the backyard flocks were stated to have contact with wild birds which have the potential to be infected with *S*. Pullorum (3). Rats and mice have been shown to act as biological vectors for *S*. Pullorum and may potentially transmit *S*. Pullorum into a backyard chicken flock (9). Obtaining birds from NPIP hatcheries shows a significant decrease in paratyphoid *Salmonella* ($p<0.05$) which is an indication that the NPIP hatchery monitoring practices for *S*. Enteritidis are effective (10).

The results from this study indicate that *Salmonella* spp. may be commonly located in backyard poultry. Backyard poultry owners need to understand that *Salmonella* spp. exist within their flock so that they can be sure to take simple measures such as washing their hands (6). This project has shown that backyard poultry owners can implement simple measures in their flocks that will help decrease the risk of *Salmonella* spp. infection, reducing not only the risk of disease in their flocks, but also zoonotic diseases.

REFERENCES

1. Burns, T. E., C. Ribble, M. McLaws, D. Kelton, and C. Stephen. Perspectives of an underrepresented stakeholder group, backyard flock owners, on poultry health and avian influenza control. J. Risk Res. 16:245-260. 2013.

2. Center for Disease Control and Prevention. Multistate Outbreaks of Human *Salmonella* Infections Linked to Live Poultry in Backyard Flocks, 2017.

3. Center for Food Security and Publinc Health. Iowa State University. Fowl Typhoid and Pullorum Disease. 2009

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

5. Derksen T., R. Lampron, R. Hauck, M. Pitesky, and R. A. Gallardo. Biosecurity Assessment and Seroprevalence of Respiratory Diseases in Backyard Poultry Flocks Located Close and Far from Commercial Premises. Avian Diseases In-Press. 2017. http://www.aaapjournals.info/doi/pdf/10.1637/11672-050917-Reg.1

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

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

8. Madsen, J. M., N.G. Zimmermann, J. Timmons, and N.L. Tablante. Avian influenza seroprevalence and biosecurity risk factors in Maryland backyard poultry: A cross-sectional study. PLOS One 8:e56851. 2013.

9. National Wildlife Health Center. Field Manual of Wildlife Diseases: Birds. Chapter 9: Salmonella. 1988.

10. USDA-APHIS, VS. National Poultry Improvement Plan Program Standards. 2017.

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

BONE CHECK LEG EVALUATION IN CHICKENS AND TURKEYS: WHAT IS NORMAL?

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SUMMARY

Commercial broiler and turkey skeletal disease and lameness are influenced by production management, nutrition and genetic selection with a goal of reduce the incidence of common skeletal diseases including rickets, tibial dyschondroplasia, and other mineral and vitamin deficiencies. While improvements have been made, lameness and skeletal problems continue to be an animal welfare concern in commercial production without complete understanding of the etiology and pathogenesis.

Some of the most common gross and histological lesions observed in lame meat birds for production today include ostochondrosis (bacterial or sterile), rotation of the hock joint (splayed leg), enterococcal spondylitis (ES) in the free thoracic vertebrae, and inflamed tendons due to viral infection. Most birds display either the femoral head and proximal femur, proximal tibia, or the free thoracic vertebrae bones are involved. There have been several studies from the University of Arkansas evaluating BCO and how it is related to lameness in birds. In addition, North Carolina State University has evaluated hatchery environments and its relationship to embryo overheating resulting in lameness during broiler grow out and there is a negative effect. Other researchers at NC State as also researched ES and vertebral osteomyelitis and the most recent etiological bacterial causing agent, *Enterococcus caecorum* (EC) and how it is related to broiler lameness.

Many skeletal challenges have been evaluated at the university level but no formal programs have been developed to monitor lameness challenges in the field especially with new enzymes being utilized like phytase. In order to assist the industry in evaluating what specific causes of lameness occur in the field within a complex, a Bone Check program has been established to evaluate bone health as it relates to lameness at all ages during grow-out. Prior to establishing this value added program, we first needed to establish a baseline of normal. This was performed by initially evaluating a broiler complex, big bird Ross 708 X Ross Yield Plus that was not currently experiencing lameness issues. A large survey of 18 commercial broiler houses ranging from 1-50 days, which were determined to have a normal gait score of "0" (AAAP Gait Scoring System) was grossly and histologically evaluated. The initial study revealed subclinical phosphorus rickets, micro-fractures (green-stick) and hemorrhaging within the femoral hip socket and long bones at various ages. The microscopic fractures, hemorrhaging and P rickets occurred predominately prior to seven days of age.

After these observations in the field of the bone lesions within the first week of life, further work was conducted with the long bones to determine if we could determine when some of these lesions occur and if they are correlated with handling or hatchery processing. After surveying three chicken complexes, we have found a low level of microfractures from birds in the hatchers through farm placement. In addition, inflammation in the long bones can seen from the pre *in ovo* process through farm placement in normal chicks. At the same time it was observed that hemorrhaging was occurring especially in the joint area, which was further investigated to determine the potential cause. There was an objective to determine if the results from the hatchery or could it be also linked to the breeders so more work has been completed.

With the initial work there was some concern where the microfractures and hemorrhaging might be occurring and could they be linked to the bird collection process. Within the initial studies chicks have been euthanized with cervical dislocation. A follow-up study was performed to compare cervical dislocation compared to a Euthasol® (Virbac Corporation, Fort Worth, TX) injection to induce euthanasia. These results suggest that some of the acute hemorrhaging could be induced during the euthanasia process; however, other lesions such as thrombi and microfractures were also accompanied by hemorrhage of longer duration. Microfractures between both groups were about the same. When lame birds are evaluated microscopically inflammation and occasionally bacteria are observed. More field work is continuing to build a profile of normal broilers and the incidence of lameness.

GENOME WIDE ANNOTATION OF CIS-REGULATORY ELEMENTS IN THE CHICKEN GENOME

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ABSTRACT

Infection and disease negatively affect performance, growth, morbidity and mortality in production animals and has major economic impacts on commercial poultry production. Specifically in poultry production, additional factors that affect commercial and economic performance need to be considered in breeding. These factors include egg laying performance or growth efficiency in broilers. Regulatory elements control the expression of genes and consequently phenotype. The ENCODE project was launched more than a decade ago with the goal of better annotating the human genome for both coding and noncoding elements. This has greatly improved our understanding of gene regulation and dramatically advanced our understanding of disease-associated genetic variants. Applying this approach to annotate the chicken genome will catapult research in agricultural animals. Epigenetic modifications such as histone tail modifications and DNA methylation are not only key to the regulation of unique transcriptome patterns, these modifications are indispensable as genome annotators to uncover cell- and tissue-specific regulatory elements. Uncovering the location of regulatory elements and determining their interactions will provide the necessary framework to understand how regulatory networks govern gene expression and how genetic and environmental influences alter these networks to impact animal growth, health and disease susceptibility or resistance. Investigation of these modifications in poultry will improve our ability to provide the tools for the poultry community at large to use the results generated to enhance our understanding avian development, immunology, and metabolism.

SUMMARY OF PROJECT

Infection and disease adversely impact performance, growth, morbidity and mortality in production animals and has major economic impacts on commercial poultry production. This is exacerbated by consumer worries about antibiotic use, which pressures producers to use alternative strategies to prevent infection. An illustrating example is the avian influenza outbreak of 2015, which has been described as the worst in US history with the loss of over 48 million birds (chicken and turkey). This event underscores the need to functionally annotating the chicken genome to understand complex genetic traits such as disease resistance, which in turn could serve as a powerful tool to ensure sustainable agricultural systems. Furthermore, in poultry production, additional factors affecting commercial and economic performance need to be considered in breeding. Factors including intestinal health, egg laying performance or growth efficiency in broilers certainly are of particular economic significance. To improve our understanding of the underlying mechanisms controlling these and other traits, identification of cis-regulatory elements on the genome is warranted. These regulatory elements control the expression of genes and consequently phenotype; however, the targeted tissues and cells for analysis need to be determined for each trait of interest, as these regulatory elements are tissue specific, and even differ from cell to cell within a given tissue (1, 2). Additionally, environmental factors such as nutrition interact with epigenetic modifiers working at these regulatory elements, thus making it possible to promote the expression of desirable productive traits by management (3).

In the past, selective breeding of poultry has resulted in improved feed conversion ratios in broilers, albeit at the cost of decreased immune competence and greater susceptibility to disease. In contrast, some lines of birds are known to be more resistant to a variety of pathogens (4, 5). The emergence of antimicrobial resistance suggests that genetic resistance to disease would provide an advantage over the use of antimicrobials. While parameters of immune competence, such as antibody production, phagocytic activity and cytokine production of the host are known, the genetic and molecular basis for disease resistance is still not well understood. It has become apparent that pathogens interact with epigenetic regulators of the host, aiding immune evasion and replication of the pathogen (6). Identification of regulatory elements and their influence on target genes directly responsible for enhanced immunity in poultry is crucial in order to select for traits that improve

disease resistance for breeding purposes and flock health.

Innate and adaptive immune responses play an important role in chicken disease resistance. Targets of functional annotation include blood monocytes and macrophages, naïve and activated T cells as well as B cells. Particularly macrophages show stronger immune responses from naturally resistant animals in response to infection (7-9). Blood macrophages from disease resistant B2 haplotypes displayed largely different gene expression patterns during differentiation as well as after IFNγ stimulation, compared to macrophages from disease susceptible B19 haplotype chicks (10). Epigenetic regulation influences macrophage differentiation and function and requires substantial reorganization of the chromatin landscape (11, 12). Knowledge of the involved regulatory elements will enable predictions of how these cells respond to stimuli, which is key to understand the innate immune response and disease resistance, traits that are fundamental to poultry production. T cells are the main effectors of cell mediated immunity in pathogen. Naïve T cells are activated to differentiate further into helper or cytotoxic T cells, and eventually memory cells. As with macrophages, epigenetic regulation at regulatory elements of T cell differentiation is an important process to further investigate. Several organs are populated by macrophages and T cells, and are involved in immune responses, such as lung, liver, kidney, thymus, spleen and intestine. Differences in immune cell populations in these tissues may account for disease resistance in some chicken lines in response to various pathogens.

Breeders have been very successful improving egg laying performance, but the genetic basis of increased egg production is not well understood. Estrogen influences development and function of the oviduct, mobilization of calcium for egg shell formation and production of egg yolk proteins. Epigenetics regulation of genes involved in steroid biosynthesis has been shown, such as CPY19A1 expression in lutein cells (13). Expression of microRNA has also been associated with a high rate of egg production in chicken ovarian follicles (14). These studies show that epigenetic regulation potentially plays a role in reproductive health and efficiency, which can be further investigated if epigenetic modifications and regulatory elements are mapped. Taken together, these studies emphasize the need for annotation of regulatory elements and the importance of understanding regulatory element function in the chicken, not only in the context of enhanced immunity and disease resistance, but intestinal and reproductive health.

The goal of this project is to leverage transcriptomic and epigenomic methods to annotate transcripts – coding and noncoding; and cis-regulatory elements in the chicken genome, including promoters, enhancers and insulators. The ENCODE project was launched more than a decade ago with the goal of better annotating the human genome for both coding and noncoding elements (15). This has greatly improved our understanding of gene regulation and dramatically advanced our understanding of disease-associated genetic variants. Applying this approach to annotate the chicken genome will catapult research in agricultural animals. Uncovering the location of regulatory elements and determining their interactions will provide the necessary framework to understand how regulatory networks govern gene expression and how genetic and environmental influences alter these networks to impact animal growth, health and disease susceptibility or resistance.

RESULTS

RNA-seq analysis. Transcription factors modulate the genetic programs within cells, therefore, identification of divergent patterns of TF expression between chicken B2 and B19 haplotype cells may provide further insight into the mechanisms underlying the diverse macrophage differentiation and activation phenotypes. B2 haplotypes are more resistant to disease than B19 haplotypes (8, 9). Among a set of 28 transcription factors expressed either highly on day -6 in B2 monocytes or highly on day -6 in B19 monocytes (Figure 1), seven were divergently expressed in B2 cells (PIWIL1, ASCL4, TFAP2E, NEFM, GCM1, and GLIS) while five were differentially expressed in B19 cells (ZNF142, GATA6, MECOM, TBX10 and FOXL2). Next, a set of 75 transcription factors (TFs) exhibiting a variety of differentially expressed patterns between B2 and B19 monocytes and macrophages prior to IFNγ stimulation was identified and hierarchically clustered in order to identity TF gene sets with shared expression patterns (Figure 1A). Such TFs may represent combinatorial members of transcriptional regulatory circuits responsible for controlling the gene expression program of specific classes, or types of macrophage related transcripts. The genetic background likely impacts this network.

Divergent miRNA Expression in B2 and B19 Macrophages Targets Distinct Genes. In addition to TFs, miRNAs provide a parallel mechanism for divergent patterns of gene expression and subsequent altered cellular phenotypes between B2 and B19 birds. A set of 59 miRNAs were identified that exhibited divergent expression patterns between the B2 and B19 haplotype cells prior to IFNγ stimulation (Figure 1B).

CONCLUSION

Infection and disease negatively affect performance, growth, morbidity and mortality in production animals and has major economic impacts on commercial poultry production. Important traits identified for poultry production are disease resistance and immunity, growth and reproductive traits, particularly in egg layers. Cell- or tissue-specific gene expression is mediated by the complex interactions between transcriptional machinery and cis-regulatory elements such as promoters, enhancers and insulators. Epigenomic approaches to study gene regulation have proven to be the most successful and most utilized tools for cis-regulatory genome annotation. Recent large-scale efforts including ENCODE, Reference Epigenome Project, modENCODE and mouseENCODE have all capitalized on the application of epigenomic assays to annotate their respective genomes of interest. To understand regulatory mechanisms of tissue-specific gene expression and the impact that genetic variations occurring at cis-regulatory elements have on health and disease of chickens, it is important to have a well-annotated genome and identify these regulatory elements. The potential long-range improvement and sustainability of U.S. agriculture and food systems will be advanced by providing the tools to improve breeding for disease resistance in poultry and subsequently prevent economic losses due to contagious pathogens and thus increasing food security.

REFERENCES

1. Gonzalez-Recio, O., Toro, M.A., Bach, A. Past, present, and future of epigenetics applied to livestock breeding. Front. Genet. 6:305. 2015

2. Shen, Y., Yue, F., McCleary, D.F., Ye, Z., Edsall, L., Kuan, S., Wagner, U., Dixon, J., Lee, L., Lobanenkov, V.V. et al. A map of the cis-regulatory sequences in the mouse genome. Nature 488(7409):116-120. 2012.

3. Burdge, G.C., Hoile, S.P., Uller, T., Thomas, N.A., Gluckman, P.D., Hanson, M.A., Lillycrop, K.A. Progressive, transgenerational changes in offspring phenotype and epigenotype following nutritional transition. PLoS One 6(11):e28282. 2011.

4. Swaggerty, C.L., Pevzner, I.Y., He, H., Genovese, K.J., Nisbet, D.J., Kaiser, P., Kogut, M.H. Selection of broilers with improved innate immune responsiveness to reduce on-farm infection by foodborne pathogens. Foodborne Pathog. Dis. 6(7):777-783. 2009.

5. Li, J., Li, R., Wang, Y., Hu, X., Zhao, Y., Li, L., Feng, C., Gu, X., Liang, F., Lamont, S.J. et al. Genome-wide DNA methylome variation in two genetically distinct chicken lines using MethylC-seq. BMC Genomics 16:851. 2015.

6. Galvan, S.C., Garcia Carranca, A., Song, J., Recillas-Targa, F. Epigenetics and animal virus infections. Front Genet 6:48. 2015.

7. Bellamy, R. Susceptibility to mycobacterial infections: the importance of host genetics. Genes. Immun. 4(1):4-11. 2003.

8. Dawes, M.E., Griggs, L.M., Collisson, E.W., Briles, W.E., Drechsler, Y. Dramatic differences in the response of macrophages from B2 and B19 MHC-defined haplotypes to interferon gamma and polyinosinic:polycytidylic acid stimulation. Poult. Sci. 93(4):830-838. 2014.

9. Collisson, E., Griggs, L., Drechsler, Y. Macrophages from disease resistant B2 haplotype chickens activate T lymphocytes more effectively than macrophages from disease susceptible B19 birds. Dev. Comp. Immunol. 67:249-256. 2017.

10. Irizarry, K.J.L., Downs, E., Bryden, R., Clark, J., Griggs, L., Kopulos, R., Boettger, C.M., Carr, T.J. Jr., Keeler, C.L., Collisson, E., Drechsler, Y. RNA sequencing demonstrates large-scale temporal dysregulation of gene expression in stimulated macrophages derived from MHC-defined chicken haplotypes. PLoS One. Aug 28;12(8):e0179391. 2017.

11. Lavin, Y., Winter, D., Blecher-Gonen, R., David, E., Keren-Shaul, H., Merad, M., Jung, S., Amit, I.: Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. Cell. 159(6):1312-1326. 2014.

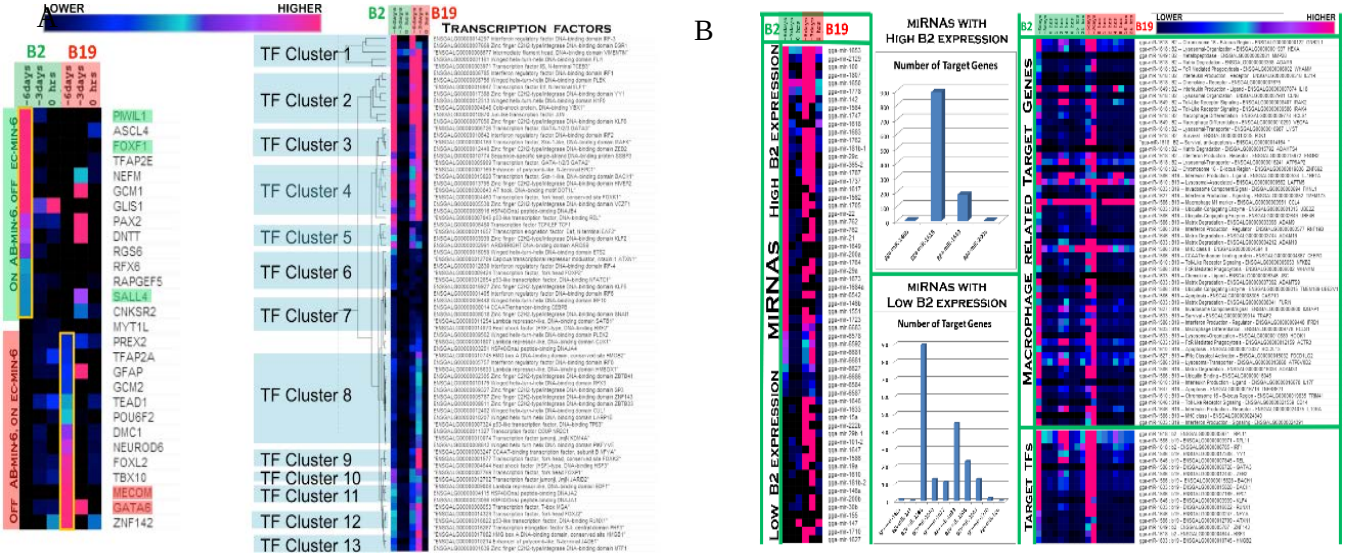
12. Gosselin, D., Link, V.M., Romanoski, C.E., Fonseca, G.J., Eichenfield, D.Z., Spann, N.J., Stender, J.D., Chun, H.B., Garner, H., Geissmann, F. et al. Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. Cell 159(6):1327-1340. 2014.

13. Zhang, X., Ho, S.M. Epigenetics meets endocrinology. J. Mol. Endocrinol. 46(1):R11-32. 2011.

14. Wu, N., Gaur, U., Zhu, Q., Chen, B., Xu, Z., Zhao, X., Yang, M., Li, D. Expressed microRNA associated with high rate of egg production in chicken ovarian follicles. Anim. Genet. 48(2):205-216. 2017.

15. The ENCODE (ENCyclopedia Of DNA Elements) Project. Science 306(5696):636-640. 2004.

Figure 1. Expression analysis B19 and B2 haplotypes. A) TFs with divergent expression in B2 versus B19 macrophages prior to IFNγ stimulation. B) miRNA with divergent gene expression in B2 and B19 Macrophages prior to IFNγ stimulation.



ATTEMPTS TOWARDS A BETTER CLASSIFICATION OF AVIAN REOVIRUS VARIANTS

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ABSTRACT

Avian reoviruses (ARVs) are extremely variable. Their variability responds to their RNA segmented genome that favors mutations and recombination events. Five and six Reovirus genotypic clusters have been described in the literature, based on the σC gene molecular characterization. In 2016 we began to molecularly characterize Reovirus variants in order to detect early genotypical divergence of ARVs causing pathology in broilers. So far, we have detected four clusters matching with the previously described clusters. In addition, we have done full genome sequencing and challenge experiments using selected isolates to confirm the σC typing and look for other genes variability in order to associate them with antigenicity and pathogenicity. The reason behind looking other genes is that σC typing in some cases fail to correlate not only with pathotypes but also with serotype and/or antigenic type. This situation could affect the effectiveness of autogenous vaccines since this typing is used for autogenous vaccine virus selection. So far, L3 and M2 genes that encode for outer capsid proteins showed to be variable and potential candidates for a more comprehensive classification. The proteins encoded by these genes might generate neutralizing antibodies and their variability might be associated with Reovirus pathology. We will propose a strategy to better characterize ARVs in order to understand pathogenic and serological determinants.

INTRODUCTION

Since 2011, the poultry industry globally has been facing the consequences of the emergence of new avian Reoviruses (ARV) variant strains. Reovirus variants induce economic losses in the affected flocks due to lack of uniformity, poor feed conversion, increased condemnations and reduced animal welfare in meat-type poultry. ARV are distributed worldwide in chickens, turkeys (1, 2) and are ubiquitous in poultry farms. However, this is not enough to attribute them the cause of a clinical disease and rigorous diagnosis is required. Recently, pathogenic variant ARV are recognized as the main cause of viral arthritis and tenosynovitis in broiler chickens and their breeders (3).

Reoviruses are non-enveloped and possess a double-stranded and segmented RNA. Their high rate of mutation and recombination allows them to escape from the vaccine generated antibodies. The viral genome consists of 10 segments that encode 12 proteins of which eight are structural. The S1 gene encodes for the σC protein, an outer capsid protein responsible for viral cell attachment. In addition, elicits neutralizing antibodies (2). Because of its characteristics, S1 has been the target of molecular characterizations.

Based on recent investigations, five (4) and six (5, 6) genotypic clusters have been described using S1 sequences for classification demonstrating a high variability of the virus and its ability to circumvent vaccine generated immunity (4). One of the problems of S1 gene typing is that in some cases it fails to correlate not only with pathotypes but also with serotype and/or antigenic type. Since this methodology is used to select viral variants for autogenous vaccine production it affects the effectiveness of these prevention tools. Preliminary studies in our laboratory have shown that other outer capsid proteins, μB and μBN coded by the M2 gene and λC protein coded by the L3 gene (9), show promising characteristics and can be used in classification schemes (7).

In this study, we will attempt to renovate the currently used molecular characterization strategy. Approximately 150 Reovirus isolates collected from 2016 to early 2018, and some historical samples will be S1 gene characterized. Based on the clinical signs attributed to the isolates in the field, strains will be

selected for a full genome screening. Serotyping and pathotyping of selected strains will follow.

MATERIAL AND METHODS

Virus isolates for characterization were selected from a list of Reovirus isolates recovered from clinical tenosynovitis at the California Animal Health and Food Safety Laboratory (CAHFS). The selection criteria was based on clinical importance, tissue of origin and CPE when isolated. These isolates were confirmed by RT-PCR through the amplification of a conserved segment of the S4 gene (11). Confirmed isolates were submitted to an S1 gene RT-PCR and sequencing using the forward and reverse primers (8). After curating the sequences and aligning those for homology determination phylogenetic trees are prepared in order to compare the sequences and group them in different genetic clusters (10). Selected isolates will be chosen for the RNA extraction and preparation of cDNA libraries and posterior full genome sequencing (Illumina Hi Seq 3000). Pathological and serological characterization of the isolates will allow us to study distinct genes association with these parameters.

RESULTS AND DISCUSSION

So far, two rounds of ARV molecular characterization have been done. Results confirm the presence of variant reoviruses in 4 out of the 6 reported genotypic clusters (Table 1). Most of the isolates from both characterizations are grouped in cluster 1 (vaccine cluster). However, homologies of these viruses to S1133 are below 78%. The rest of the isolates are grouped in clusters 2, 3 and 4 and their homologies to S1133 were below 58.9, 57.5 and 54.8% respectively (Table 1). Seven selected isolates were full genome sequenced. The analysis of these sequences has shown a high variability on L3 and M2 being candidates for further molecular characterization (Table 2).

We are currently working on the S1 characterization of a third batch of viruses. After that, more viruses will be selected for full genome studies. These results will be discussed.

(This work will be submitted for publication in *Avian Diseases*.)

REFERENCES

1. Rosenberger, J. K., Sterner, F. J., Botts, S., Lee, K. P., & Margolin, A. In vitro and in vivo characterization of avian reoviruses. I. Pathogenicity and antigenic relatedness of several avian reovirus isolates. Avian diseases, 535-544. 1989.

2. Jones, R. C. Reovirus infections. Diseases of Poultry, 13th ed. (Swayne, DE, Glisson, JR, McDougald, LR, Nolan, LK, Suarez, DL and Nair, VL eds.), John Wiley and Sons, Inc., Ames, 89-107. 2013.

3. Van der Heide, L. The history of avian reovirus. Avian diseases, 44(3), 638-641. 2000.

4. Sellers, H. S. Current limitations in control of viral arthritis and tenosynovitis caused by avian reoviruses in commercial poultry. Veterinary microbiology, 206, 152-156. 2017.

5. Lu, H., Tang, Y., Dunn, P. A., Wallner-Pendleton, E. A., Lin, L., & Knoll, E. A. Isolation and molecular characterization of newly emerging avian reovirus variants and novel strains in Pennsylvania, USA, 2011–2014. Scientific reports, 5. 2015.

6. Ayalew, L. E., Gupta, A., Fricke, J., Ahmed, K. A., Popowich, S., Lockerbie, B., & Gomis, S. Phenotypic, genotypic and antigenic characterization of emerging avian reoviruses isolated from clinical cases of arthritis in broilers in Saskatchewan, Canada. Scientific Reports, 7(1), 3565. 2017.

7. Benavente, J., & Martínez-Costas, J. Avian reovirus: structure and biology. Virus research, 123(2), 105-119. 2007.

8. Kant A., F. Balk, L. Born, D. van Roozelar, J. Heijmans, A. Gielkens and A. ter Huurne. Classification of Dutch and German avian reoviruses by sequencing the σC protein. Veterinay Research. 34:203-212. 2003.

9. Gallardo, R.A., Hauck, R., Corsiglia, C., Senties-Cue, G., Shivaprasad, H. L., & Crossley B. Molecular Epidemiology of Clinically Relevant Reoviruses in California. Proceedings of the 66th Western Poultry Disease Conference. Sacramento, California, USA. 44-46. 2017.

10. Gallardo R.A., Hauck R., Crispo M., Figueroa A., Senties-Cue G.C, Stoute S., Shivaprasad H.L., Corsiglia C., Crossley B. Molecular Characterization as a Surveillance Strategy for Clinically Relevant Reoviruses. AAAP Symposium, Indianapolis, IN. 2017.

11. Bruhn, S., Bruckner, L., & Ottiger, H. P. Application of RT-PCR for the detection of avian reovirus contamination in avian viral vaccines. Journal of virological methods, 123(2), 179-186. 2005.

Table 1. Sequence genotypic cluster distribution and homology to S1133 for the two molecular characterizations

Genotypic cluster	# Sequences		Homology to S1133	
	1st characteriza: on	2nd characteriza: on	1st characteriza: on	2nd characteriza: on
1	16/21	20/26	74.22 to 77.82%	73 to 76.6%
2	2/21	-	57.74 to 58.89%	-
3	1/21	1/26	57.53%	55.7%
4	2/21	5/26	53.35 to 54.81%	51.5 to 53.6%

Table 2. Full genome S1133 homology chart. Eight selected isolates were compared gene by gene against a full genome sequence from the vaccine strain S1133. *Still under analysis (10).

Isolate	L 1	L 2	L 3	M1	M2	M3	S1	S2	S3	S4
CA-K-4	88.1	90.1	72.4	89.4	75.5	81.6	81.3	90.3	85.9	81.9
CA-K-3	88.1	90.1	73.1	89.5	75.6	81.5	81.1	90.3	86	81.9
CA-K-12	88.7	83.8	72.4	87.3	*	87.6	38.4	91.9	85.5	80.7
CA-K-2	89.2	89.5	72.9	88.4	*	89.5	59.3	91.6	89.1	80.2
CA-T-9	88.7	83.8	72.3	87.2	*	87.7	38.3	91.7	85.6	82
CA-T-1	88.1	90.1	73.1	89.6	75.5	81.7	81.2	90.3	85.9	81.9
CA-T-4	88.1	90.1	73	89.5	75.5	81.6	80.8	90.3	86.8	81.9
Proteins	γA I. Core	γB Core	γC I.C- O. Cap.	μA I. Cap.	μB O. Cap.	μNS N.S.	σC minor cap.*	σA I. Core*	σB O. Cap.	O. Cap.

PERSISTENCE OF LPAI AND HPAI IN TREATED AND REUSED POULTRY LITTER

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INTRODUCTION

Between 2014 and 2015, the largest and most expensive outbreak of animal disease in the United States spread throughout the Midwest, affecting 21 states and causing the deaths of almost 50 million birds (2, 4). The virus behind the outbreak, a highly pathogenic strain of avian influenza (HPAI), infected poultry in both the U.S. and Canada, costing up to three billion dollars in economic losses. HPAI inflicted almost 100% mortality in poultry and challenged control and containment measures established by the commercial poultry industry (4). Spreading via waterfowl migratory pathways, avian influenza viruses (AIV) have caused outbreaks worldwide, devastating the poultry industry and in some occurrences, creating major human health issues (1). Numerous investigations associated with the host, wild waterfowl as disease vectors, and the environment have been conducted. However, research into the biology of AIV inside commercial poultry barns is scarce. Understanding the interactions between footbaths, bedding material and other factors from the commercial poultry setting are critical to find effective preventative and biosecurity tactics.

Previous research has investigated the persistence of low pathogenic avian influenza (LPAI) and HPAI in the bedding material of broilers, turkeys and layer feces (3). Thus, the current study focuses on analyzing the influence of treated and reused litter on the persistence of LPAI and HPAI viruses. The reuse of poultry litter is a common practice worldwide to reduce costs of production and for environmental sustainability (12, 10). Although there are concerns over the reuse of litter, performance results from chickens reared on reused litter did not differ from chickens that were raised on new litter (6, 8, 5).

MATERIALS AND METHODS

In this study, broiler litter of one and 11 cycles as well as turkey litter of one and four cycles were used and spiked with either LPAI or HPAI virus. Negative controls were crafted using a mix of all cycled broiler litter and a mix of all cycled turkey litter, both uninfected. Samples were collected from all litter samples prior to contamination and every 12 hours until 96 hours post contamination. Presence of LPAI and HPAI from all litter samples was analyzed by RT-qPCR, virus isolation was performed to retrieve live viral particles.

RESULTS AND DISCUSSION

As we reported previously (3), HPAI was observed to persist longer, up to 60 hours, compared to LPAI, which persisted up to 36 hours in broiler litter (Figure 1). Results showed higher viral loads on both viruses in turkey litter, no differences in live virus recovery by isolation was detected between cycle numbers in broiler and turkey litter, indicating that LPAI and HPAI persisted equally in each litter regardless of cycle number.

Sodium bisulfate products are commonly used as litter acidifier between cycles to reduce atmospheric ammonia (11). These products have reduced the prevalence of *E. coli*, salmonella, and campylobacter in poultry premises (9, 7). However, its antiviral capabilities have not yet been investigated. Even though the litter used in this experiment was treated these products between cycles we did not see much effect of it on the persistence of avian influenza. We are currently working on experiments to analyze the true effect of PLT on LPAI.

REFERENCES

1. Capua, I. and S. Marangon, Control of Avian Influenza in Poultry. Emerging Infectious Diseases, 2006. 12(9): p. 1319-1324.

2. Dargatz, D., et al., Case Series of Turkey Farms from the H5N2 Highly Pathogenic Avian Influenza Outbreak in the United States During 2015. Avian Dis, 2016. 60(2): p. 467-72.

3. Hauck, R., et al., Persistence of Highly Pathogenic and Low Pathogenic Avian Influenza Viruses in Footbaths and Poultry Manure. Avian Diseases, 2017. 61(1): p. 64-69.

4. Hubbard, L.E., et al., Highlighting the complexities of a groundwater pilot study during an avian influenza outbreak: Methods, lessons learned, and select contaminant results. Environ Res, 2017. 158: p. 212-224.

5. Jones, F.T. and W.M. Hagler, Observations on New and Reused Litter for Growing Broilers. Poultry Science, 1983. 62(1): p. 175-179.

6. Kennard, D.C. and V.D. Chamberlin, Growth and Mortality of Chickens as Affected by the Floor Litter. Poultry Science, 1951. 30(1): p. 47-54.

7. Line, J.E., Campylobacter and Salmonella populations associated with chickens raised on acidified litter. Poult Sci, 2002. 81(10): p. 1473-7.

8. McCartney, M.G., Effect of Type of Housing and Litter on Production of Broilers. Poultry Science, 1971. 50(4): p. 1200-+.

9. Pope, M.J. and T.E. Cherry, An evaluation of the presence of pathogens on broilers raised on poultry litter treatment-treated litter. Poult Sci, 2000. 79(9): p. 1351-5.

10. Roll, V.F.B., M.A.D. Pra, and A.P. Roll, Research on Salmonella in broiler litter reused for up to 14 consecutive flocks. Poultry Science, 2011. 90(10): p. 2257-2262.

11. Terzich, M., et al., Effect of Poultry Litter Treatment(R) (PLT(R)) on the development of respiratory tract lesions in broilers. Avian Pathol, 1998. 27(6): p. 566-9.

12. Thaxton, Y.V., C.L. Balzli, and J.D. Tankson, Relationship of broiler flock numbers to litter microflora. Journal of Applied Poultry Research, 2003. 12(1): p. 81-84.

Figure 1. Live virus recovery by isolation in SPF eggs from litter samples in experimental replicates 1 and 2. POS stands for live virus recovered and NEG for no virus recovered.

Hours post spike	B1		B11		T1		T4	
	LP	HP	LP	HP	LP	HP	LP	HP
0	POS	POS	POS	POS	POS	POS	POS	POS
12	POS	POS	NEG	POS	NEG	POS	NEG	POS
24	POS	POS	NEG	POS	NEG	POS	NEG	POS
36	POS	POS	NEG	POS	NEG	POS	NEG	POS
48	NEG	POS	NEG	POS	NEG	POS	NEG	POS
60	NEG	POS	NEG	POS	NEG	POS	NEG	NEG
72	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
84	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
96	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Hours post spike	B1		B11		T1		T4	
	LP	HP	LP	HP	LP	HP	LP	HP
0	POS	POS	POS	POS	POS	POS	POS	POS
12	POS	POS	POS	POS	POS	POS	POS	POS
24	POS	POS	NEG	POS	NEG	POS	NEG	POS
36	NEG	POS	NEG	POS	NEG	POS	NEG	POS
48	NEG	POS	NEG	POS	NEG	POS	NEG	POS
60	NEG	POS	NEG	POS	NEG	NEG	NEG	POS
72	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
84	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
96	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

THE IMPACT OF COCCIDIOSIS IN MINOR POULTRY SPECIES

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SUMMARY

Typically chickens and turkeys are the primary source of poultry protein; some other sources are waterfowls, guinea fowls and pigeons. The US waterfowl production had increased and this mirrors the global upward trend. Pigeon rearing is also becoming a profitable business. The eggs and flesh of guinea fowls are consumed due to the good nutritional qualities such as tenderness, leanness, high in essential fatty acids and vitamins, and low in cholesterol. The husbandry practices in these industries are similar to those of the chicken and turkey industries; however, on a smaller scale. The disease challenges in the minor poultry species are similar to the chicken and turkey industries. Coccidiosis is caused by protozoa of the phylum Apicomplexan, family Eimeriidae. Following the ingestion of infective coccidian oocysts several asexual replications and a sexual replication occur within the host epithelial cells. These processes occur over five to seven days depending on the coccidia species. Extensive damage to the intestinal mucosa may occur during the replication processes and may lead to impaired host performance. Coccidia are generally host-specific and some species are site specific. Signs of coccidiosis may include ruffled feathers, decreased growth rate, severe watery, mucoid and or bloody diarrhea, and mortality. Gross lesions may not always be apparent but for those species that cause gross lesions these are distinctive in location and appearance and are diagnostic.

Coccidiosis in ducks may involve moderate to heavy mortality. Clinical and subclinical coccidiosis appears to be common and can produce morbidity, poor performance and mortality. Coccidia in ducks may be caused by one of three genera (*Eimeria*, *Wenyonella* or *Tyzzeria).* Signs of coccidiosis are anorexia, weight loss, weakness, distress, morbidity, and up to 70% mortality. Coccidiosis in young pigeons is severe and mortality may be 15-70%. However, the disease is less severe in older birds and subclinical infections may persist for long periods. The most common species of coccidia in pigeons is *E. labbeana* (Labbe 1896) Pinto 1928. Common signs of infection are anorexia, greenish diarrhea, marked dehydration, and emaciation. Droppings may be tinged with blood and the entire digestive tract may be affected.

Recently there have been some concerns about coccidiosis outbreaks in guineas that are reared in confinement. There are no drugs approved for coccidiosis control or live coccidia vaccines for guineas. This report is an attempt to understand coccidiosis in young keets and the control with selected anti-coccidials that have been approved for other gallinaceous birds. The signs of coccidiosis were watery droppings, feces with high uric acid content, ruffled feathers, huddling and unthrifty. The most effective drugs were Robenz and Zoamix in which there was a complete elimination of oocysts release in the feces. Deccox and Nicarb had marginal control whereas they allowed 40-50% of the oocysts to complete lifecycle and released oocysts in the feces. Amprol was poor, in that it allowed more than 70% of the oocysts to complete their lifecycle and release oocysts in the feces.

REFERENCES

McDougald, L. R. and S.H. Fitz-Coy. Coccidiosis, In: Diseases of Poultry, 12th ed. Y.M. Saif, ed. Blackwell Publishing, Iowa pp1068-1085. 2008

UNDERSTANDING CORYZA OUTBREAKS, PERSISTENCE, AND MOLECULAR BIOLOGY

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INTRODUCTION

Changes in productive systems, particularly in layer flocks, from cages to cage free housing have set new rules in terms of management, biosecurity and disease prevention. In addition, the multifactorial nature of diseases in poultry add complexity to the diagnosis, prevention and control. From January to September 2017, 54 cases of infectious coryza (IC) caused by *Avibacterium paragallinarum* have been diagnosed by the California Animal Health and Food Safety Laboratory System (CAHFS). Those cases clinically presented a chronic course associated with mortality caused by peritonitis and increased condemnations in broilers and in layers severe drops in egg production and peritonitis. More than 50% of those cases had concurrent infections with infectious bronchitis virus (IBV). The first goal of this investigation was to understand if the coryza agent, *Avibacterium paragallinarum* was persistent in the environment. In addition, we aimed to generate a reliable molecular characterization method to use when serological typing is not an option.

MATERIALS AND METHODS

A bacterial persistence experiment was carried out. Fifteen *Avibacterium* positive birds, confirmed by culture or PCR, were placed with contaminated field bedding material from infected farms inside six isolators in order to contaminate the environment with the bacteria (Figure 1). After four days, birds were euthanized and *Avibacterium* naïve broilers were introduced at different time points from 12 hours up to 96 hours. Exposed birds were swabbed for Avibacterium PCR four and seven days post exposure (DPE) and necropsied at seven DPE. Additionally, seeder and exposed birds were sampled for *Gallibacterium anatis* PCR (Figure 2).

Conventionally, *Avibacterium* isolates are typed using two systems described by Page (1) and KUME (2). Since serological typing was not available, we attempted a molecular characterization of three isolates, one from broilers and two from layers using full genome next generation sequencing (NGS). We focused the analysis and characterization on the HMTP 210 and Hag A genes. Those genes encode the Hemagglutinin protein on the *Avibacterium paragallinarum*. Since hemagglutination activity is used for serotyping, we thought this was the best approach. Results from genotyping were compared with sequences in GenBank that possessed serotype information.

RESULTS AND DISCUSSION

After two to three days mortality was seen in seeder birds. Gross pathology indicated polyserositis from which only one third were positive to *Avibacterium* by culture. After seeders were euthanized we detected an erratic presence of the *Avibacterium* by culture and PCR. In addition, we found *Gallibacterium* by PCR in one third of the birds. Contact birds, before exposure, were tested by PCR showing to be negative to *Avibacterium* but positive to *Gallibacterium*. Swabs were collected at four and seven days post exposure, all birds showed to be negative to *Avibacterium* but approximately one third of birds were positive to *Gallibacterium*. A considerable amount of the exposed birds showed clinical signs related with swollen heads, mucous exudate and respiratory signs. At histopathology, mild or moderate lesions were detected in the upper respiratory tract.

These results suggest that *Avibacterium paragallinarum* was involved in the primary disease, other potential pathogens such as IBV and or *Gallibacterium anatis* were detected. Finally, we can conclude that *Avibacterium paragallinarum* did not persist in the isolators.

In regard to typing, we focused on the HMTP 210 and Hag A genes because these are the genes that code

for the hemagglutinin protein of the bacteria. In addition, they determine pathogenicity and immunogenicity. The three isolates showed to be very similar, only one single nucleotide polymorphism was detected in the broiler isolate. When the HMTP 210 gene was investigated, a close homology (100%) was detected when isolates were compared to the Modesto (C-2) and the H18 (C-1) strain. When Hag A was compared a closer homology was detected with C-1 rather than C-2 strains.

Our molecular typing did not show a major molecular shift of the field isolates compared with the vaccine strains. It might be a synergistic effect with other pathogens such as IBV or Gallibacterium that might explain the clinical outcomes of this infection. This topic requires further investigation. Since the vaccines available in the State only have the serotype C-2 it would be interesting to compare the effectiveness of C-1 vaccines to challenges with the field isolates. Is important to emphasize that proper vaccination protocols and techniques are needed and that bacterins always require a booster.

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

REFERENCES

1. Page, L.A. *Haemophilus* infections in chickens. 1. Characteristics of 12 *Haemophilus* isolates recovered from diseased chickens. Am. J. Vet. Res. 23:85-95. 1962.

2. Kume, K., A. Sawata, T. Nakai, and M. Matsumoto. Serological classification of Haemophilus paragallinarum with a hemagglutinin system. J. Clin. Microbiol. 17:958-964. 1983.

Figure 1. Experimental design used to seed the isolators environment.

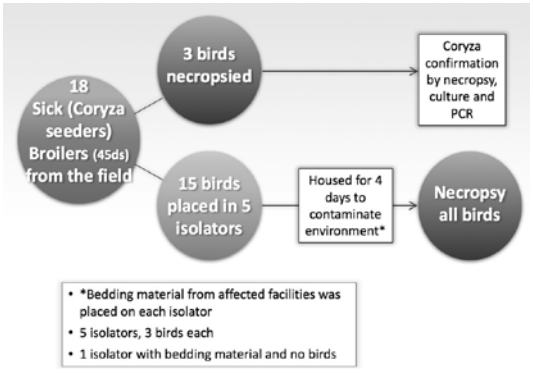
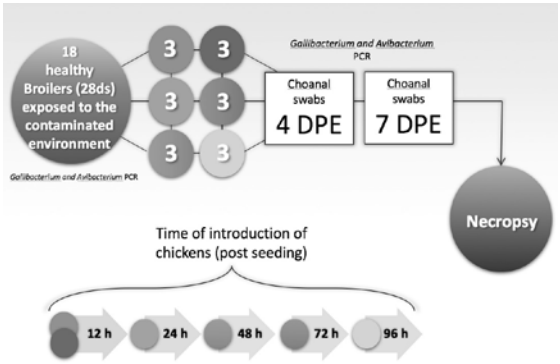


Figure 2. Experimental design for the exposure of the naïve birds to an *Avibacterium* contaminated environment.



USING POULTRY AS A MODEL TO IMPROVE FOOD SECURITY IN RURAL NICARAGUA

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INTRODUCTION

Residents of Sabana Grande (SG) are primarily subsistence farmers who depend on livestock for food and economic security. Ninety-six percent of families own chickens, making poultry the most common food species within SG. However, disease burden and lack of management result in unpredictable production and high mortality in the community's flocks. Historically, few families monitor and vaccinate for disease or have appropriate biosecurity practices. Together, these factors have made poultry an unreliable food source.

With improved preventative care and education, we believe poultry production can be increased sustainably, improving food security for many families in SG. Since 2012, we have educated the community on preventive measures to reduce poultry disease while improving management and biosecurity. These training sessions resulted in the vaccination against ND of a high percentage of all poultry in SG. ND is the most common burden of poultry production in endemic zones of the world causing up to 100% mortality in infected flocks (1). Low literacy rates are a challenge in SG. In many households only children are literate. Furthermore, our information does not seem to be shared beyond the 15-20 families that regularly attend workshops and brainstorming sessions. Children's education is valued in the community. In addition, they adapt quickly to new programs and foster future leadership potential, so they can be a valuable resource for instituting sustainable community change (2).

For the first time, we targeted our training to primary school children ages. Veterinary medicine students from the U.S. were trained by education experts at our school in strategies towards hosting poultry workshops to children between six and 12 years old. These students trained Nicaraguan veterinary students and a community youth group to facilitate three activities. These activities focused on concepts of disease transmission, poultry disease prevention, biosecurity, and risk assessment.

We evaluated our importance in the community poultry network using a Social Network Analysis (SNA). We hypothesized that introducing poultry husbandry skills in the classroom will affect changes at the family level and improve food security in SG in a sustainable manner.

MATERIALS AND METHODS

Three stage training. Nicaraguan and U.S. veterinary medicine students were trained by education and poultry medicine experts on education activities to address poultry health and management. Veterinary students trained a local youth group with interest in agriculture to be facilitators of these activities, and finally elementary school students participated in the activities under the supervision of all the previously mentioned groups.

Activities. Three activities adapted from a prepared curriculum for 4-H groups in the U.S. covering concepts of disease transmission, poultry disease prevention, biosecurity and risk assessment were carried out with the elementary students.

Data collection. A risk assessment tool was taken home by each of the students to be filled out by them with the help of their parents. In addition, we asked them to work on a risk mitigation plan for their flock covering the information provided during the activities.

A SNA was performed to the parents of the elementary students prior to the experiential learning activities.

RESULTS AND DISCUSSION

Risk assessment. All 23 students and their families worked on a risk assessment exercise for poultry. Even though, people in the community understood how to prevent diseases in poultry (75%), only 63% vaccinate against NDV, the most important cause of poultry mortality in SG.

More than 80% of respondents introduce poultry to their flocks, while only 43% always use quarantine procedures. This is a topic in which further training is

needed since one of the most common ways of introducing disease into a flock is the introduction of foreign birds.

Risk mitigation plans. Eighteen out of 23 students (78.3%) worked on the risk mitigation plan. Elementary school students reported a diverse range of options to improve health and management of their flocks. Every student brainstormed three measures. The most common risk mitigation strategies were: water management 22.6%, building a coop 19.4%, feed improvement 16%, vaccination and quarantine capabilities 9.7% each, waterfowl deterrent measures and fomite avoidance 6.5% each, and carcass, mosquito and waste management 3.2% each (Fig. 1).

Social Network Analysis (SNA). In order to evaluate the poultry networks in the community and understand where the poultry information is obtained, the families of the elementary school students were asked the question: "Where do you get reliable poultry information?". Interestingly, veterinarians are consulted to get reliable poultry information. UCD SVM represents the second most important reliable source of information (Table 2).

In conclusion, the risk assessment tool and SNA demonstrated the effectiveness of our work, specifically in terms of NDV prevention in poultry through vaccination. The risk mitigation plans created by the students reflected the topics and material covered in the learning activities. The language barrier was a secondary factor during the training. Finally, the Nicaraguan veterinary students and the community youth group reduced the cultural barrier between elementary school students and our UCD veterinary students.

REFERENCES

1. Alexander D.J., Bell J.G., and Alders R.G. 2004. Technology Review: Newcastle Disease, with special emphasis on its effect on village chickens. Rome. FAO Animal Production and Health Paper #161.

2. Oluwakemi, L., S. Olaide, and O. Ajoke. 2011. Children's Participation in Agricultural Activities in the Adopted Villages of the Institute of Agricultural Research and Training, Nigeria. Journal of Rural Social Sciences 26 (2): 126-36.

Figure 1. Risk mitigation strategies mentioned by students after training sessions.

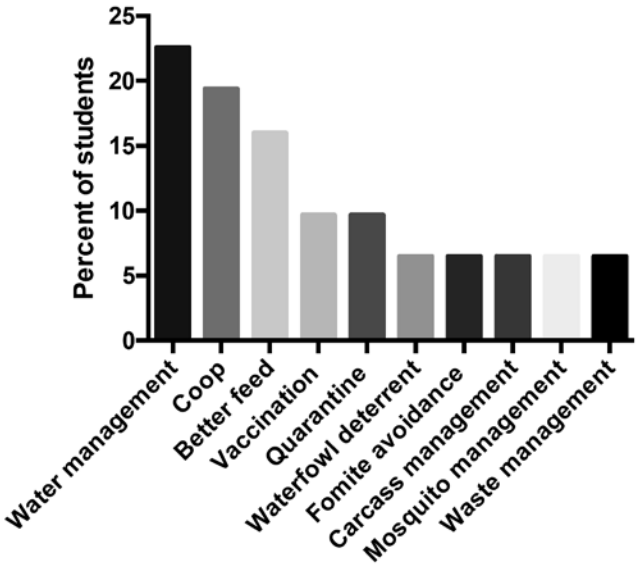
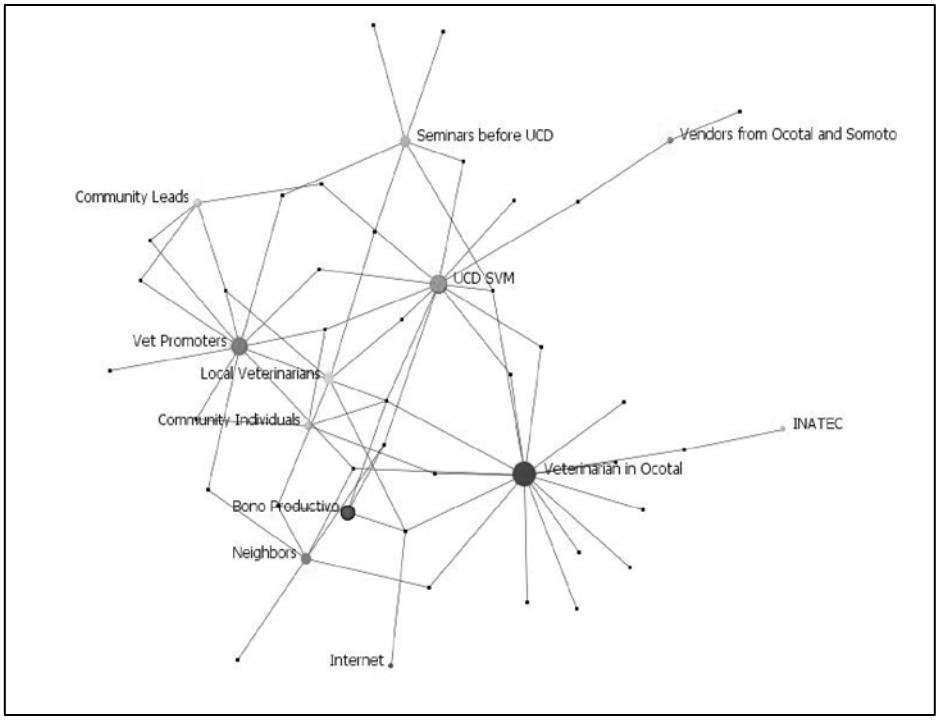


Table 2. SNA graph showing the networks established between the community and poultry information sources. Size of the dots represents centrality index.



TURKEY HEALTH AND DISEASE – FIELD SITUATIONS LINKED TO IMMUNOSUPPRESSION

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DISCUSSION

I appreciate the opportunity to hopefully stimulate some research interest for turkeys in general and immunosuppression and disease in particular. Over the last decade, virtually all expensive turkey research has been dedicated to food safety, antimicrobial resistance, welfare, or other catchy topics. This has left minimal funding for any complex or expensive conditions affecting the health of turkeys themselves – and research into immunosuppression in long-lived costly species under field conditions is expensive and time-consuming. It requires somewhat specialized facilities, interested personnel, geographic proximity and ready access to the turkeys affected. That said, my personal opinion remains that virtually anything interfering with optimal turkey performance will be at least somewhat immunosuppressive due to diversion of metabolic resources required for efficient growth.

Since the topic relates to field experiences, there will be no references cited – PubMed is readily available, and for the reasons mentioned in the previous paragraph there is virtually nothing published on this subject involving modern commercial strains of turkeys. This renders most of the available published work essentially irrelevant to modern field conditions – the birds and the business are evolving faster than the literature.

Those factors that have been examined to see if they cause immunosuppression in turkeys have uniformly caused some degree of immunosuppression, so the easiest answer to the question is that everything examined so far causes some degree of immunosuppression in turkeys. From a field standpoint, it looks like there are many factors – mycotoxins, bordetellosis, hemorrhagic enteritis, mycoplasmosis, atmospheric ammonia, genetics, chilling, coccidiosis, sexual maturity, transportation, inappetance, incubation conditions, PEMS (poult enteritis mortality syndrome), and stress. This is not a complete list, just one that probably won't cause much argument among those familiar with the older literature.

Mycotoxins. There is a large body of literature on the effects of various mycotoxins on different parts of the immune system, usually in laboratory *in vivo* or *in vitro* systems using purified individual toxins, but not much in turkeys. This is rather different than the field situation, which may involve periodic or sustained exposure to multiple toxins, probably in combination(s) at various stages of the birds' growth. This is further compounded by the probable existence of a variety of mycotoxins and mycotoxin metabolites that have yet to be characterized – if analyses for known mycotoxins are negative, does that mean none are there? If exposure to a mycotoxin causes changes in immunomodulation in the laboratory or *in vitro*, does that have practical meaning in the field? No one knows. The best mycotoxin prevention is excellent grain quality, if possible.

Bordetellosis. The early literature for *Bordetella avium* (previously *Alcaligenes faecalis*) demonstrated some degree of interference with immunity. However, those strains also caused significant tracheal cartilage damage. I haven't seen that in some years, and recent genotyping demonstrates significant genetic change in modern strains of bordetella compared to historic strains. Are modern strains more/less immunosuppressive? No one knows, but probably…..

Hemorrhagic enteritis. A prominent cause of splenic damage as well as the (now) more subtle intestinal damage – I haven't seen significant duodenal hemorrhage in over 20 years. Colibacillosis frequently follows clinical disease even in the absence of overt intestinal damage – an argument for immunosuppression, but again, no research has been done in modern strains of turkey with currently available field or vaccine strains of the disease. Are we vaccinating too much or too little? Are the currently available vaccine concoctions protective, of adequate titer, or being administered correctly? There is no test for live virus in splenic vaccine as far as I know due to cytotoxic effects of splenic components, and the performance of tissue culture-derived vaccines remains erratic. The AGID and PCR tests frequently used for potency will detect both live and dead virus in vaccines…. The argument over the significance of HE maternal antibody in turkeys under field conditions continues, especially from those more familiar with the significance of maternal antibody in other fowl under laboratory conditions…. No one knows, personally I believe it irrelevant under field conditions. Maternal antibody transfer in turkeys

doesn't appear to be particularly efficient compared to that in chickens.

Atmospheric ammonia. Literature in the 1980s suggests that 25 ppm is immunosuppressive in turkeys. I am unaware of anything more recent. Modern strains now weigh almost twice what the turkeys of the early 80s did at almost the same age – certainly the toms I see in the field are uncomfortable at lower levels – what's going on with the immune system in these modern birds? No one knows.

Genetics. For years, the demand has been get to bigger birds with more white meat rather than development of markets for dark meat. Fine as long as cardiopulmonary, hepatic, gastrointestinal, and immune capacity keep pace. I'm not at all convinced that they have. Primary turkey breeders are understandably constrained to small groups of birds by the nature of their business. Selection conditions now more closely mimic commercial densities, but not commercial diets or population numbers. But are selection parameters including heart/lung/liver/GI to body weight ratios or immunological function tests? We certainly see differences in vaccine responses and field challenges between strains. At what point will the obsession with breast meat yield begin to interfere with livability or other welfare considerations? - Keeping in mind that once it does, it will take 5-7 years to effectively address the problem…hopefully the wizards know what's happening here – I don't. It won't be pretty if livability in AgriStats starts to decline, which would provide somewhat deserved ammunition to the anti-animal agriculture and Luddite communities.

Chilling/inappetance. These are probably closely related if turkeys are chilled enough they don't eat. Whether chilled turkeys that maintain consumption are immunosuppressed due to production of stress hormones specifically is unknown. Turkeys that don't eat for a wide variety of reasons seem to have more problems with colibacillosis, but the exact mechanism is not known to me. Could be changes in intestinal integrity, an increase in stress hormones, failure to maintain metabolic heat production associated with rapid growth – no one knows. Sexual maturity (puberty) – An occurrence in the turkey industry that does not occur in the meat chicken industry. Most heavy turkeys are sexually mature for at least the last third of the growing period, depending on strain. Certainly steroid hormones have been demonstrated to be immunosuppressive, and certainly a number of problems begin to manifest themselves post-puberty in modern turkeys. How many of these problems (osteomyelitis, aspergillosis, cellulitis) are related to rapid growth, management conditions, or some degree of immunosuppression? – no one knows. These conditions themselves are probably immunosuppressive, but again, no one knows.

Incubation conditions. About 40 years ago, the hatch window on poults began shifting earlier rather quickly, probably due to genetic changes. Many hatchery managers did not respond, resulting in a large number of dehydrated, overheated poults being produced that did not thrive in the field. An interested embryologist actually assessed humoral immune response in poults removed from the hatcher at appropriate times vs. those removed on schedule and demonstrated a significant reduction in response to administration of killed antigens. Hatchery management has improved since then, although the continued use of multi-stage incubation systems, variations in egg size and inability to control and respond to egg inventories and storage appropriately continue to hamper efforts to prevent immunosuppressive events due to incubation conditions – primarily associated with overheating. Good work has been done in this area in chickens – very little in turkeys.

Other diseases. Mycoplasmosis, PEMS, turkey coronaviral enteritis – again, all have been associated with immunosuppression in the past. Other diseases are probably likely immunosuppressive as well. Generally, turkeys seem to lack agents known to specifically target immune tissues – I know of nothing in turkeys similar to infectious bursal disease in chickens. HE certainly affects the spleen as described earlier, but agents specifically affecting thymus, bone marrow or bursae of turkeys remain essentially unexamined and/or without known remedy beyond non-specific management interventions.

There is no readily available field test for immune function in turkeys beyond crude assays for humoral immunity. This handicaps evaluation of its importance under commercial conditions and virtually no institutional research is being conducted to examine it. There are very few institutions performing research actually benefitting turkeys themselves rather than addressing the broader societal concerns of antimicrobial resistance, animal welfare, and food safety. One could easily justify inclusion of examination of immunocompetence within those areas, but as far as I know, this is not being done, which is a shame. Immunocompromised birds probably excrete more bacteria important in food safety, probably have more disease and mortality affecting welfare, and probably require more use of antibiotics to control secondary infections, which could contribute to antibiotic resistance.

More modern genotyping techniques could also assist in agent identification, but then work would be required to determine actual etiological association so possible preventive measures could be examined.

Where would one send turkey bursae to determine what agents may be present? Who then could determine etiological significance and possible preventive measures?

Interesting issues – if you have an interest, give me a call/email. Maybe we can actually move forward in a directed manner.

SERUM, LUNG AND SPLEEN METABOLOMICS PROFILE ANALYSIS TO INVESTIGATE MECHANISM OF CPG-ODN MEDIATED IMMUNE MODULATION IN BROILER CHICKENS

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SUMMARY

The metabolome refers to the complete set of small molecules found in a biological sample, cell, organ or tissue. Metabolomics analysis assists in understanding the changes occurring in the metabolome of a living organ or tissue due to disease, medication, immunization or stress. We have previously shown that oligodeoxynucleotides containing CpG motifs (CpG-ODN) stimulate innate immunity and protect neonatal broiler chickens against *Escherichia coli* septicemia. In this study, we show a previously unrecognized phenomenon that metabolomics profiles change in tissues and serum with the administration of CpG-ODNs. In our experiments, 11-day-old broiler chickens were divided into two groups; one group was administered CpG-ODN intramuscularly (50 µg/50µL/bird), and the control group was administered saline (50uL). Blood samples (n=5) were collected 3 hours, 24 hourrs, and 72 hours post-administration. Spleen and lung samples (n=5) were collected 24 hourrs and 72 hours post administration of CpG-ODN or saline. Serum was separated from the blood, and tissue samples were prepared for metabolomics analysis. Nuclear Magnetic Resonance (NMR) Spectroscopy and Direct Injection Liquid Chromatography Mass Spectrometry (DI/LC-MS/MS) techniques were applied on the samples to identify metabolites. Analysis of metabolomics data was conducted using MetaboAnalyst 3 software. We observed that the predominant metabolites differentiating between the CpG-ODN and saline control groups involved the key metabolites in energy production in glycolytic, ketogenic, citrate cycle and fatty acid oxidation pathways. Projection of these metabolites on metabolic pathways suggested that the energy production pathways have changed. It is tempting to speculate that such changes in energy pathways may influence the cellular and molecular recruitment caused by CpG-ODNs. We believe that the metabolomics profile analysis will further our understanding of biochemical pathways associated with CpG-ODN-mediated immunomodulation and identifying specific metabolite(s) contributing to the change.

INTRODUCTION

The first week of life is a crucial period for neonatal chicks as they are susceptible to many bacterial and viral infections due to their naïve immune system. Bacterial infections such as *Escherichia coli* and *Salmonella* lead to increased mortality during the first week causing massive losses (8). As a result, the chicken industry is constantly looking for novel methods to prevent such infections. It was previously discovered that intramuscular (IM) administration and *inovo* injection of CpG-ODN are able to significantly protect neonatal broiler chicks against bacterial infections (2,6). Further on it was reported that this protection is primarily mediated by a Th-1 biased immune response which results in the activation and recruitment of immune cells (5). As part of the quest to explore the mechanism of CpG-ODN mediated immunomodulation, we were interested in identifying specific markers that could provide us clues regarding this immune response.

Metabolomic analysis is a newly emerging field of “omics” research. In this analysis, the complete set of small molecule metabolites (<1500kDa) found in a specific cell, organ or organism known as the “metabolome” is identified and quantified using high throughput techniques (7). Metabolites have been described as early reporters of diseases as they are directly related to different pathways of pathogenesis (1) making metabolomics a powerful tool of predicting and understanding mechanisms. There are limited studies that explore the metabolome of chickens or birds at the moment(4). As a result we were eager to utilize this technique with the objective of identifying specific metabolic markers that could predict CpG-ODN mediated immune stimulatory mechanism.

MATERIALS AND METHOD

Experimental design. This work was approved by the Animal Research Ethics Board, University of Saskatchewan and adhered to the guidelines by the Canadian Council on Animal Care. Day-old broiler chicks were obtained from a local hatchery and placed in an animal isolation room at the Animal Care Unit, Western College of Veterinary Medicine. Water and commercial broiler ration were provided *ad libitum*. At day-11 post-hatch they were divided into two groups; one group was administered intramuscularly CpG-ODN (50 μg/50μL/bird) and the control group was administered saline (50 L). Blood samples (n=5) were collected 3hours, 24 hours, and 72 hours post-administration from each group. Spleen and lung samples (n=5) were collected 24 hours and 72 hours post administration of CpG-ODN or saline. Serum was separated from the blood, and tissue samples were prepared for metabolomics analysis. The metabolomic analysis was performed using Nuclear Magnetic Resonance (NMR) Spectroscopy and Direct Injection Liquid Chromatography Mass Spectrometry (DI/LC-MS/MS) techniques where a total of 160 metabolite levels were identified. Analysis of metabolomics data was conducted using MetaboAnalyst 3 software.

Metabolomics data analysis. From the serum samples, 130 metabolites and from the lung and spleen samples, 87 metabolites were identified above the limit of detection (LOD) and used for further analysis to discover their significance in CpG mediated immunomodulation. For the solid organ samples, data were corrected for wet lung and spleen weight before the extraction of the samples. Data from each sample type were log transformed and pareto scaled. Principal Component Analysis (PCA) was performed for all sample types whereas Partial Least Squares – Discriminant Analysis (PLS-DA) was also done for the serum metabolites. Unpaired comparisons as well as paired comparisons were done between CpG and control groups for each time point for each sample type. Pathway Enrichment Analysis was performed to identify the biochemical pathways in chickens which might be involved with the key metabolites identified in the study. Then those metabolites were projected on the identified metabolic pathways to develop biochemical hypothesis.

RESULTS

In terms of serum metabolite levels, metabolome of CpG-ODN received birds clearly differed from the control birds at 3 hours and 24 hours post treatment. However at 72 hours, the difference was less apparent due to an increase within group variability, particularly in the control group. Overall, the key metabolites that were identified as top contributors to the difference were acetone, myo-inositol, dimethylamine, serotonin and malonate which were upregulated in CpG-ODN birds and 3-OH butyrate, glutamine, pyruvate and alanine that were down regulated. These differences were at statistically significant levels.

In the lung samples, considering the PCA, there was a slight separation between CpG-ODN and control group using Proximal Component 2 (PC 2) and Proximal Component 5 (PC 5) at 24 hours following CpG-ODN administration. Due to higher within group variation, only three metabolites were identified as causing a significant difference which are glucose (high in CpG-ODN birds), adenine and uracil (low in CpG-ODN birds). At 72 hours, a slight separation was observed using PC 2 and PC 3. Again, due to higher within group variability, only two metabolites; lysine (high in CpG-ODN birds) and acetic acid (low in CpG-ODN birds) significantly differed between the treatments.

Spleen samples showed a slight separation between CpG-ODN and control groups at PC2 and PC4. However, due to high within group variability, no interesting metabolites were identified as contributors to the difference. At 72 hours, there was no separation between CpG-ODN and control groups even when using the best combinations of PCs.

According to the pathway enrichment analysis it was evident that the key metabolites differentiating were involved with energy production pathways.

CONCLUSIONS AND DISCUSSION

Administration of CpG-ODN certainly changed the serum, lung and splenic metabolomes in chickens. Based on the pathway enrichment analysis we can conclude that the key metabolites identified as contributors to this difference are involved in energy production pathways. Serum is the best sample to use for this type of study as it allows sampling of the same bird at different time points. In this study, serum samples showed less within group variability compared to lung or spleen samples. Therefore, serum samples demonstrated clear difference in the metabolic profile of CpG treated and untreated groups than solid tissue samples. One reason could be due to the less vigorous sample preparation procedure applied to serum samples than tissue samples. Other factors such as wet-weight to dry-weight differences and the presence of blood and other impurities in tissue samples could have affected the metabolite levels in the lung and spleen. Caution will be taken in future during sample collection to minimize such confounding factors and a bigger sample size will be used to reduce the effect of within group variation. It's

particularly interesting to see the changing energy metabolites upon the administration of CpG-ODN. Based on our previous findings of the cellular activation and recruitment in CpG treated chicks(3), we can conclude that the changes in the metabolic profile maybe associated with the cellular and molecular changes occurring as a result of CpG-ODN mediated immunomodulation.

REFERENCES

1. Clish, C. B. Metabolomics: an emerging but powerful tool for precision medicine. Cold Spring Harb. Mol. Case Stud. 1. 2015.

2. Gomis, S., L. Babiuk, B. Allan, P. Willson, E. Waters, N. Ambrose, R. Hecker, and A. Potter. Protection of Neonatal Chicks Against a Lethal Challenge of Escherichia coli Using DNA Containing Cytosine-Phosphodiester-Guanine Motifs. Avian Dis. 48: 813–822. 2004.

3. Goonewardene, K. B., S. Popowich, T. Gunawardana, A. Gupta, S. Kurukulasuriya, R. Karunarathna, B. Chow-Lockerbie, K. A. Ahmed, S. K. Tikoo, M. Foldvari, P. Willson, and S. Gomis. Intrapulmonary Delivery of CpG-ODN Microdroplets Provides Protection Against Escherichia coli Septicemia in Neonatal Broiler Chickens. Avian Dis. 61: 503–511. 2017.

4. Le Roy, C. I., L. J. Mappley, R. M. La Ragione, M. J. Woodward, and S. P. Claus. NMR-based metabolic characterization of chicken tissues and biofluids: a model for avian research. Metabolomics 12. 2016.

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

6. Taghavi, A., B. Allan, G. Mutwiri, A. Van Kessel, P. Willson, L. Babiuk, A. Potter, and S. Gomis. Protection of Neonatal Broiler Chicks Against Salmonella Typhimurium Septicemia by DNA Containing CpG Motifs. Avian Dis. 52: 398–406. 2008.

7. Wishart, D. S. Current Progress in computational metabolomics. Brief. Bioinform. 8: 279–293. 2007.

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

QUALITY CONTROL AUDITS OF KILLED VACCINATION

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SUMMARY

Vaccination programs for breeders and egg layers include vaccination with inactivated vaccines. Killed (inactivated) vaccines are used to prevent disease in the vaccinated bird and/or to provide maternal antibody for protection of progeny. Killed vaccines are often more costly than live organism vaccines and in addition the labour cost to administer killed vaccines can be more expensive than the vaccine, both of which are important reasons to vaccinate correctly. But even more important is that incorrect immunisation exposes the vaccinated birds or their progeny to disease, with resultant welfare consequences and economic loss. Also killed vaccines can cause serious injuries to staff if accidentally injected. Hence it is important for efficacy, welfare, safety and economic reasons that killed vaccines are administered safely and correctly. Audits of Quality Control Points of vaccination with killed vaccines on 16 farms revealed considerable variability in results between farms and indicated to management where improvements in vaccination procedures should be implemented. It was recommended that killed vaccination audits should be undertaken within poultry companies as part of the Quality Control process to manage the efficacy and safety of killed vaccinations.

INTRODUCTION

Killed vaccines are included in vaccination programs of breeders and commercial egg layers. Effective immunisation with killed vaccines requires: a technically sound vaccination program appropriate for the flock and the infectious disease status in the area; correct receipt, storage, transport, preparation, and administration of killed vaccines; auditing of vaccination procedures; and serological monitoring, where appropriate, to confirm that the vaccination program and procedures are likely to be effective. Quality Control audits of killed vaccination were undertaken in 16 flocks to assist poultry companies manage immunisation of their flocks.

MATERIALS AND METHODS

Killed vaccination audits. Sixteen audits of vaccination into breast muscle with killed vaccines containing antigens of infectious bursal disease (IBD), Newcastle disease (ND), egg drop syndrome (EDS), *Salmonella* Types B+C+E, *Pasteurella multocida* (fowl cholera) or *Avibacterium paragallinarum* (infectious coryza) were conducted over a two-year period for six of Australia's major chicken meat companies, two audits for Australia's two major egg layer breeder companies and an audit for one chicken meat breeder company in New Zealand. Each audit of the Australian chicken meat companies was undertaken in different regional divisions of the company throughout Australia.

Quality control procedures used for monitoring killed vaccination.

- Store vaccine in an alarmed refrigerator at 2-8 ºC. Do not freeze. Check for "separated emulsion"
- Remove vaccine from the refrigerator the day before vaccination and hold in an air-conditioned room overnight to allow the vaccine to reach 15-25 ºC prior to injection or warm to 25 ºC in a water bath immediately before using
- Transport vaccine from storage rooms to farms/houses without ice bricks to retain temperature at 15-25 ºC
- Add blue dye (MSD VacTrace for bird injection) to at least one container of vaccine per vaccinator
- Shake vaccine containers vigorously before and during use for thorough mixing
- Calibrate the vaccination gun by injecting at least 10 doses into a syringe or measuring cylinder to confirm the correct dose of the injection
- Use new needles every 1000-2000 birds
- Observe vaccine administration to confirm that the vaccine is being injected correctly into the fleshiest part of the breast and safely to prevent "needle-stick" injuries to staff
- Vaccinate at least 1000 birds, including all sex errors and culls, with the blue coloured vaccine to obtain 20 sex errors and culls for post-mortem examination

- Euthanize and post-mortem the vaccinated sex errors and culls with the vaccination crew present to confirm that the vaccine has been deposited correctly into the breast muscle
- Reconcile the number of doses used against the number of birds vaccinated
- Discuss any improvements to the vaccination procedures that need to be implemented with the vaccination supervisor and poultry company management
- Blood test at least 20 birds/house six weeks following vaccination for IBD, ND or EDS, as appropriate

RESULTS

Audit of vaccination quality control points. There was some variability in the standard of the vaccination procedures which could influence the effectiveness and safety of the vaccination. Vaccination crews were always interested in observing the results of their vaccinations and were often surprised at the results. Storage of vaccine was generally satisfactory. Only one farm did not have an alarmed refrigerator. Eleven farms did not warm vaccine to 15-25 ºC, as per label instructions, to prevent cold shock to the bird, to prevent intense local reactions and to reduce viscosity of the vaccine to ensure an even flow through the vaccination gun needle thus generating a full dose for each bird to optimise the effectiveness of the vaccine. Six farms did not mix the vaccine sufficiently to ensure that each bird is injected with a full dose of vaccine. Eight farms did not calibrate the vaccination gun. Seven farms did not change needles frequently, with resultant bruising and leakage of vaccine onto the breast muscle surface or into feathers in some cases. Nine farms did not adequately protect against “needle stick” injuries to staff. Thirteen farms did not vaccinate all birds correctly into the breast muscle. A summary of the results is given in Table 1.

Serological monitoring. Relevant serological monitoring was either not undertaken or the results were not available. Achievable mean ELISA titers for IBD virus are >12,000 with a CV <20% at 4 to 6 weeks after killed vaccination in chicken meat breeders to provide protection of progeny against infection with Australian standard or Australian variant IBD viruses that can cause immunosuppression in the first two weeks of life. At 4 to 6 weeks following killed vaccination of breeders and egg layers, achievable mean HI titers for ND are >8 (log 2) and for EDS >6 (log 2).

DISCUSSION

Auditing killed vaccination, as described in this paper, revealed that there were deficiencies in vaccination procedures which could result in lack of efficacy in vaccinated birds or protection of progeny against immunosuppression and which could jeopardise the safety of staff. Interestingly, vaccination crews usually considered that they were vaccinating effectively and safely and were very surprised when audits showed that this was not the case. Killed vaccinations are an integral component of vaccination programs for long-lived chickens and as such can have a major influence on the performance or liveability of the vaccinated birds or their progeny.

Correct procedures used for vaccination and monitoring of these procedures are essential for flock health, bird welfare and profitable poultry production. The audits undertaken in Australasia are also applicable in other countries, with likely resultant improvements in killed vaccination effectiveness and flock performance.

Table 1. Detailed results of quality control points for vaccination.

Company/ Farm	Alarmed/ Fridge	Injected 15-25C	Mixed Well	Gun calibrated	New Needle <2000 birds	Injected safely	Injected correctly*
A/1	✓	X	X	✓	✓	✓	✓ (0/5)
A/2	✓	✓	✓	✓	✓	✓	X (8/18)
A/3	✓	X	✓	X	X	X	X (1/16)
A/4	✓	X	✓	✓	✓	✓	X (15/25)
B/1	✓	X	✓	✓	✓	✓	X (3/17)
B/2	✓	✓	✓	X	X	✓	X (10/30)
B/3	✓	X	✓	✓	X	X	X (7/17)
B/4	✓	✓	X	✓	✓	X	✓ (0/20)
C/1	✓	X	✓	✓	✓	✓	✓ (0/6)
C/2	✓	X	✓	✓	✓	✓	X (1/6)
D/1	✓	✓	X	X	X	X	X (8/25)
E/1	✓	X	✓	X	✓	X	X (3/10)
E/2	X	X	✓	X	X	X	X (2/17)
F/1	✓	X	X	X	X	X	X (10/18)
F/2	✓	X	X	X	X	X	X (5/14)
G/1	✓	X	X	X	✓	X	X (6/40)

*Number of birds not correctly injected/total number of birds autopsied

DEMOGRAPHIC CHARACTERISTICS OF SMALL POULTRY FLOCKS IN ONTARIO

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SUMMARY

The recent increase in the number of small flocks throughout Ontario calls for an assessment of their demographic characteristics in order to evaluate the potential risk they may pose to commercial poultry operations. A prospective surveillance study of small flock postmortem submissions to the Animal Health Laboratory was conducted over a two year period (October 2015 – September 2017). Completion of a standardized husbandry and biosecurity questionnaire was a requirement of the submission.

Of 160 submissions, chickens were the most common species of bird submitted, followed by turkeys, gamebirds, and waterfowl. The primary reason for raising birds was personal consumption, other reasons reported include pets, farm gate sales, and breeding stock. Birds were acquired from various sources, including on-line classifieds, hatcheries, friends, and feed stores. Birds were often kept on the premises for less than one year, although periods of longer than five years were reported. Birds were frequently housed in mixed groups. Most birds had some free-range access. Flock owners dealt with dead birds in several ways, with burial and incineration being most common. Over half the owners reported that the birds had not been vaccinated at the hatchery and the use of medication was not uncommon. Overall, biosecurity practices were inconsistent or inadequate.

The results of this study provide a baseline characterization of small poultry flocks throughout Ontario, and will aid in detecting specific opportunities to improve the health of small flocks and mitigate the risk they may pose to commercial poultry flocks and public health.

INTRODUCTION

In countries with a well-established poultry industry, such as Canada, there has been a substantial increase in the number of non-commercial poultry flocks raised in urban and peri-urban areas (i.e., backyard poultry) (2). In Canada, poultry production is regulated by a quota system: in Ontario non-commercial poultry operations can house up to 300 broilers, 100 layers, and 50 turkeys (4,5). Across the province of Ontario, non-commercial poultry owners can voluntarily register in a poultry organization (such as the Chicken Farmers of Ontario, CFO) at the time of purchase of birds from brokers / dealers, or hatcheries. According to the CFO, in 2014 over 14,000 non-commercial poultry growers registered their flocks (3). However, a precise number of non-commercial flocks through Ontario is not available, also considering that keeping these flocks may not be legal in larger cities, such as within Toronto's city limits (11).

Several published surveys from the United States have shown that non-commercial poultry flocks are often characterized by relaxed biosecurity practices, poor veterinary supervision and lack of prophylactic measures (vaccination) (6, 9, 10). Similarly, the few data available for Ontario have shown that a substantial amount of non-commercial poultry growers do not adopt good biosecurity practices to avoid contact with wild birds, which are potential reservoir of many poultry pathogens (1). For these reasons, non-commercial poultry flocks are generally considered to be at higher risk of contracting infectious diseases and are seen as a possible threat for spreading pathogens to commercial poultry operations.

Non-commercial flocks also represent a concern for public health, by increasing the risk of human exposure to zoonotic and foodborne pathogens (e.g., campylobacteriosis) (7). In Ontario, poultry meat and eggs that are not eligible to be sold to third parties can be consumed by the owner and his family, without veterinary inspection (8), a practice that could increase the risk of food-borne diseases in households that keep poultry for domestic consumption.

Given these potential threats to animal and human health, and the recent increase in the number of small flocks throughout Ontario, a better assessment of the demographic characteristics of these flocks has become a research priority. Understanding the composition, scope for raising, and husbandry

methods of these flocks is a first step to estimate the potential risk that they may pose to commercial poultry operations, as well as transmission of zoonotic diseases. Further, a better understanding of the characteristics of these flocks will help in estimating the need and scope of educational material directed towards small flock poultry keepers.

METHODS

A prospective surveillance study of small flock (non-quota, non-commercial) postmortem submissions to the Animal Health Laboratory was conducted over a two year period (October 2015 – September 2017). Upon the owner's consent and payment of a $25 fee, each postmortem submission was associated with a standardized husbandry and biosecurity questionnaire. The questionnaire had a total of 40 questions and included two sections. One section specifically pertained to information about the submission (30 questions), including questions about flock composition, reason for keeping birds, type of coop, biosecurity measures, and use of medications. One section had general questions regarding the premises (10 questions), such as presence of wild bird feeders, bodies of water, and contact with wild birds. Options for answers included a mix of multiple choice, yes/no, and short open answers.

RESULTS

A total of 160 submissions with associated 158 questionnaires were received (two submissions were received from the same owner). Chickens were the most commonly submitted species (83.8%), followed by turkeys (6.3%), gamebirds (5%) and waterfowl (5%). The most common primary reported reason for raising birds (answers were not commonly exclusive) included self-consumption of meat and / or eggs (70.3%), keeping as pets (38.6%), and farm gate sales of eggs (17.1%). Owners reported to have kept birds in the premises for as little as 0.1 to a maximum 120 months (median, 9 months).

Birds were reported to be acquired from various sources, including hatcheries (38.2%), friends (29.9%), feed stores (15.9%), while other sources (e.g., on-line classifieds, local breeders, etc.) accounted for another 40% of the answers. Birds were frequently housed in mixed groups (38.6%), for instance different bird types (broilers and layers) and species (chickens and turkeys) in the same coop. Only 31.65% of owners reported to keep birds in an exclusively inside coop / barn, while 63.9% had some type of free-range access. In only in 16.77% of questionnaires owners reported that birds were vaccinated at hatchery, while the rest provided a negative answer (49.85%) or were unsure (33.6%). Lack of on-farm vaccination (i.e., additional to hatchery vaccination) was reported in 97.5% of questionnaires (in the remainder of questionnaires, an answer was not provided). Less than 40% of owners reported to use antibiotics (37.3%), coccidiosis prevention (15.2%), dewormers (21.5%), and medication for external parasites (14.56%).

Overall, biosecurity practices were inconsistent or inadequate, with only 21.2% and 37.8% of owners reporting to use dedicated clothing or footwear to enter the barn, respectively. In 47.6% and 94% of answers, owners reported to wash hands before and after entering the coop, and only 1.9% responded to use a foot bath when entering the coop. In 62.4% of questionnaires, owners answered to allow visitors, and of these 6.2% only reported to have dedicated clothing for them. Only 77.4% of owners isolated sick birds.

Wild bird feeders were reported to be present within the premises in 52.9% of questionnaires. Owners answered to live near water bodies in 19.8% of questionnaires, and to use a well or the municipal system as a source of water in 81.9% and 17.4% of responses.

CONCLUSION

The goal of this study was to assess the basic demographic characteristics (flock composition, reason for raising, husbandry practices, source of birds, etc.) of small poultry flocks (non-commercial / non-quota) throughout Ontario. The results of these questionnaires were associated with a postmortem submission, and correlation between demographic data and pathology / microbiology results will be reported elsewhere. To the authors' knowledge, such a detailed study is the first to characterize the small flock population in Ontario.

From the submitted questionnaires, it appears that the source of birds, flock composition, premise layout, and husbandry methods are variable parameters among these flocks, making it difficult to capture general trends. For instance, birds were received from several sources, such as hatcheries, co-ops, friends, non-specified breeders or even classified ads (e.g., Kijiji). Chickens were the most commonly kept species, and self-consumption was a common reason for raising these birds. This could mirror the fact that many flocks in our study derived from rural areas. The high prevalence of reported self-consumption highlights the zoonotic potential in these flocks, as eggs and meat used for self-consumption are not mandated to undergo veterinary inspection, according to Canadian laws.

Overall, biosecurity measures were inadequate. We found that several husbandry practices (or lack

thereof) and premise layout may have increased the risk of disease transmission. For instance, outside access (free-range) and presence of bird feeders and bodies of water in the premises could have increased the risk of contact with wild birds, while raising birds in barns with multiple types of birds (different species, or difference commodities, such as broilers and layers) may have increased the risk of disease transmission between bird groups. Few owners were aware of hatchery vaccination, and no owner reported to have vaccinated birds after purchase (i.e., on farm vaccination). Lack of prophylactic measures might also reflect an overall hesitance of owners to use veterinary services for their flock.

Limitations of this study included a certain degree of sampling bias. As Southern Ontario was overrepresented, this might have been the consequence of geographic proximity to the diagnostic laboratories that performed the necropsy (two locations: Guelph and Kemptville, ON). Additionally, as enrollment in the study included a small fee as well as agreement to submit the questionnaire, we might have selected for motivated owners that are more knowledgeable about poultry raising than the average small flock poultry keeper. However, if that were to be the case, the overall poor biosecurity practices that were reported strongly suggest the need for educational material for these flock keepers.

This study should be considered as an initial step in understanding the different types and characteristics of small flocks present in Ontario. Ultimately, the findings derived from this study will aid in detecting specific opportunities to improve the health of small flocks and mitigate the risk they may pose to commercial poultry flocks and public health.

ACKNOWLEDGEMENTS

This project was funded (or partially funded) by the OMAFRA-University of Guelph Strategic Partnership, under the Disease Surveillance Plan, which is a joint federal-provincial Growing Forward 2 project.

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

REFERENCES

1. Buregeya, J.M., S. McEwen, J. Neil, T. Baker, G.K. Zellen. Biosecurity Practices and Geospatial Map of Ontario Backyard Poultry Flocks. *Journal of Environmental Science and Engineering (JESE)* 2:694-701(2013).

2. CBC News (2010): "The backyard chicken debate" [Internet]. Available from: http://www.cbc.ca/news/the-backyard-chicken-debate-1.916153.

3. CFO (Chicken Farmers of Ontario) - Family Food Program [Internet]. Available from: https://www.ontariochicken.ca/getattachment/aefc4851-adbc-4bfc-ba96-e900146c422b/Family-Food-Regulation.aspx

4. CFO (Chicken Farmers of Ontario). Regulation No. 2228-2008, Small Flock and Farm Gate Marketing [Internet]. Available from: https://www.ontariochicken.ca/Programs/FamilyFoodProgram.aspx.

5. CFO (Chicken Farmers of Ontario). Quota Policy No. 170-2005 [Internet]. Available from: https://www.ontariochicken.ca/Farmer-Member-Resources/Quota-Info.aspx.

6. Elkhoraibi, C., R.A. Blatchford, M.E. Pitesky, J.A. Mench. Backyard chickens in the United States: A survey of flock owners. *Poultry Science* 93:2920-31 (2014).

7. Grunkemeyer, V.L. Zoonoses, Public Health, and the Backyard Poultry Flock. *Vet Clin Exot Anim* 14:477-90 (2011).

8. OMAFRA: "Your responsibilities under the Meat Regulation" [Internet]. Available from: http://www.omafra.gov.on.ca/english/food/inspection/meatinsp/resp-under-meat.htm.

9. Madsen, J.M., N.G. Zimmermann, J. Timmons, N.L. Tablante. Evaluation of Maryland Backyard Flocks and Biosecurity Practices. *Avian Dis* 57:223 (2013).

10. Smith, E.I., J.S. Reis, A.E. Hill, K.E. Slota, R.S. Miller, K.E. Bjork, K.L. Pabilonia. Epidemiologic Characterization of Colorado Backyard Bird Flocks. *Avian Dis* 56(2):263 (2012).

11. Yonge Street Media [Internet]: "Toronto's backyard bylaw isn't eggs-actly effective, and urban poultry lovers are crying". Available from: http://www.yongestreetmedia.ca/features/backyardchickens05082013.aspx.

INVESTIGATION OF FECAL SAMPLES FROM CHICKEN BACKYARD FLOCKS FOR PARASITES

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SUMMARY

Over the last few years, there has been an increased interest in people owning backyard chicken flocks for a variety of reasons. Some enjoy having the chickens as pets, while others use them as a food source for their meat or eggs. Regardless of their reasons, most owners tend to lack the knowledge of proper biosecurity measures and risk of disease transmission (2). This can become a problem where backyard flocks become reservoirs for pathogens relevant for commercial poultry (1). These might include coccidia or flagellate parasites and potentially zoonotic *Cryptosporidium* species or *Blastocystis* species. Although the growth in backyard flock ownership is increasing, there is still very minimal information available about these flocks due to them being privately owned. Of the information that is available, there is a noticeable gap in research of parasites that infect backyard flocks. From a 2011 study of backyard chicken mortality in California, it is shown that at least 5% of chicken mortality was due to parasitic disease alone and many others to have secondary parasitic infection – coccidiosis or *Heterakis gallinarum* (3). This illustrates the need for more information on parasitic disease in backyard chickens.

Forty-seven fecal samples collected from 41 backyard chicken flocks across the state of Alabama were included in the present investigation. Oocyst counts and nematode egg counts were determined using a McMaster chamber. Of the 47 samples, coccidia were detected in 27 of the samples with up to more than 100,000 oocysts/g (opg). The average was 7,530 opg, the median 1.000 opg. Overall, the number of parasites seen in the backyard flocks were much lower compared to that of commercial poultry. Samples from different groups of chickens from the same owner showed varying levels of coccidia. The species of the coccidia in the sample were determined by deep sequencing a fragment of the cytochrome oxidase I gene (COXI) and the 18S rRNA gene. In eight of the samples, there were eggs of *Ascaridia* spp. or *H. gallinarum* and eggs of *Capillaria* spp. were detected in nine samples. Nematode egg counts were up to 1700/g for *Ascaridia* sp. and *H. gallinarum* and up to 3700/g for *Capillaria* spp. In addition to counts, the samples were tested for the presence of *Histomonas meleagridis*, *Cryptosporidium* spp., *Blastocystis* spp., and other flagellates by PCR.

Data from this study gives insight into the variety of parasites that can be found in backyard flocks. The results also show that the infection pressure with coccidia species is lower than that of commercial broiler flocks, probably due to lower stocking densities and development of immunity in older birds.

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

REFERENCES

1. Derksen, T., R. Lampron, R. Hauck, M. Pitesky, and R. A. Gallardo. Biosecurity assessment and seroprevalence of respiratory diseases in backyard poultry flocks located close and far from commercial premises. Avian Dis. accepted.

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

3. Mete, A., F. Giannitti, B. Barr, L. Woods, and M. Anderson. Causes of mortality in backyard chickens in northern California: 2007–2011. Avian Dis. 57: 311–315. 2013.

A CASE OF BACTERIAL BEAK NECROSIS IN ORGANIC COMMERCIAL LAYERS

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INTRODUCTION

Beak trimming is a common practice in commercial layers to decrease cannibalism, feather pecking, and vent pecking (1). Mechanical, hot blade, electric, and infrared are the techniques utilized to accomplish this. Trimming frequently occurs at the hatchery where cautery and gauges are used to ensure the tip of the upper mandible is trimmed (1). Improper beak trims can lead to misaligned beaks, bubbles, abnormally short beaks, or exposure of vital tissues. This case stresses the importance of proper beak trimming and sanitation.

CASE HISTORY

Four 15-week-old HyLine Brown layers presented to Purdue University's Animal Disease Diagnostic Laboratory for physical examination and necropsy. The producer submitted these birds following a history of bottom beak decay in the present and past flock, reduced feed intake, and a slight increase in mortality. This flock was organic and beak trims were performed on site by a trimming machine and crew that traveled to numerous barns. Following trimming completion of the flock, the machine was disassembled and cleaned with hot water.

Gross necropsy. On presentation, the lower mandible was dry, dark brown to black, crusty, severely shortened and easily crumbled (Figure 1). The tissue surrounding the lower mandible was inflamed (Figure 1). Saliva stained the anterior feathers of the neck. The remaining beak tissue was easily sloughed during cervical dislocation. In addition to the lower mandibular necrosis, gross necropsy found empty crops in all birds and bile staining of the proventriculus and ventriculus. There was little to no body fat in the subcutis, around the heart, and in the abdominal cavity with prominent keel bones and decreased muscle mass in the breast and thigh region (Figure 2). These findings were suggestive of a low feed intake, likely due to the condition (necrosis and loss) of the beak and mandible.

Histopathology. The stratified squamous epithelium of the beak and oral cavity was focally effaced by thick, irregular and disorganized hypereosinophilic necrotic cellular debris. Multifocally on the surface of the debris and within the debris were multifocal, randomly scattered pockets of bacteria. Along the border of the necrotic debris and subepithelium were moderate numbers of heterophils. The subepithelium was diffusely congested with edema, mild amounts of fibrin and hemorrhage, moderate inflammatory infiltrates (lymphocytes, plasma cells, macrophages, and heterophils) and focal areas of granulation tissue. Heterophils bordered the mandibular bone but did not infiltrate the bone tissue. Lymphocytes and plasma cells were multifocally located between muscle bundles.

Further diagnostics. Samples of lower mandible submitted to bacteriology cultured *Proteus* spp., *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus hyicus*. Many *Eimeria* spp. were identified by parasitology from a cecal fecal sample submission.

DISCUSSION

The lesions noted in the beak and oral cavity indicated an inflammatory process that lead to necrosis and ulceration. The bacteria isolated from the beak tissue are found in the environment and/or are normal flora found in the intestines or on the skin (2, 3). These bacteria were not the initial injury, but rather a secondary invasion following an insult or traumatic event to the beak. Possible causes included improper beak trims, mycotoxicosis, penetrating foreign bodies or caustic agents (1). Due to the history of the flock, appearance of the beaks and rule outs, the inciting cause was deemed to be improper beak trimming. This case stresses the importance of proper beak trimming and sanitation.

REFERENCES

1. Saif, Y. M., Fadly, A. M., Glisson, J. R., McDougald, L. R., Nolan, L. K., & D. E. Swayne. *Diseases of Poultry*. 12th ed., Blackwell Publishing, 2008.

2. Takeuchi, Shotaro, et al. "Isolation and Some Properties of Staphylococcus hyicus subsp. Hyicus from Pigs, Chickens and Cows." *The Japanese Journal of Veterinary Science*, vol. 47, no. 5, 18 June 1985, pp. 841-843.

3. Timoney, John F., et al. *Hagan and Bruner's Microbiology and Infecitous Diseases of Domestic*

Animals. 8th ed., Comstock Publishing Associates, 1988.

Figure 1: Photograph of the lower mandible. The tissue is severely shortened, crusty, and the surrounding tissues are inflamed.

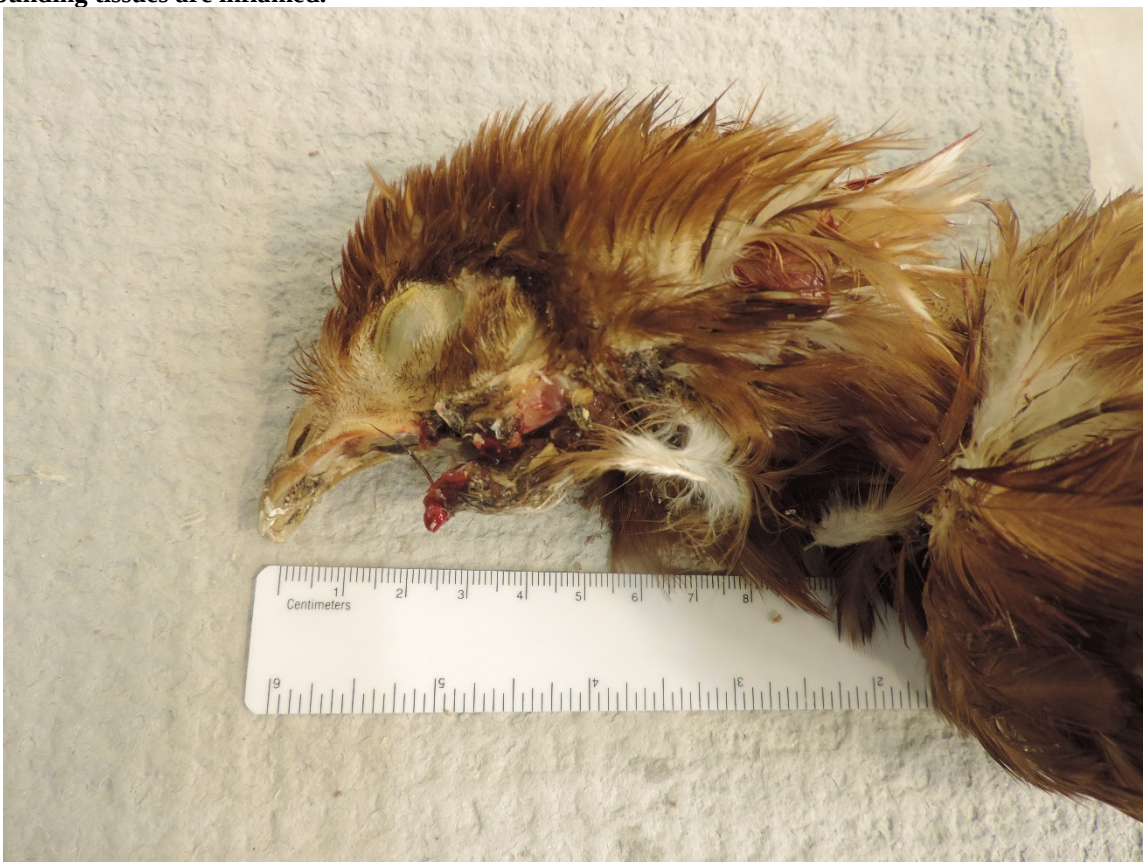
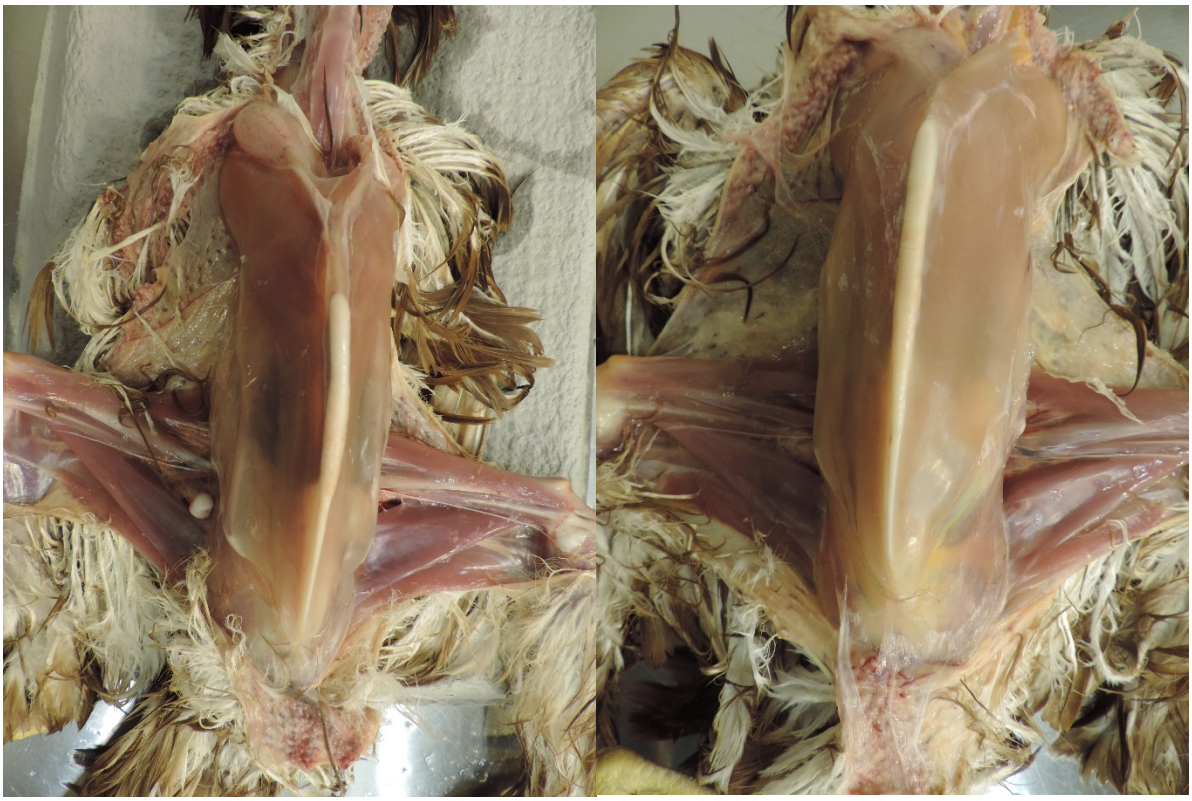


Figure 2 & 3: Photograph of pectoral muscles. There is significant muscle atrophy and a prominent keel.



ETIOLOGIES ASSOCIATED WITH BROILER CHICKEN GIZZARD EROSIONS: A REVIEW PROVIDING A FRAME TO SOLVE A COMPLEX PROBLEM

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ABSTRACT

The presence of gizzard erosions in broiler chickens is a condition that has been studied extensively by the poultry industry, however an evaluation comparing traditionally raised broiler chicks and antibiotic free broiler chicks has yet to be explored. Demands for raising broilers in an antibiotic-free management has forced the industry to pay close attention. A recent field visit revealed gizzard erosions associated with multiple flocks of antibiotic free broilers. That field observation prompted this study to compare broiler flocks raised without antibiotics and conventionally raised were evaluated at bi-weekly intervals. Gross evaluations of the gizzards were determined with the use of surface area, color, koilin involvement and gizzard muscular layer involvement with the descriptions of the lesions recorded, photographed and tabulated prior to a histomorphometric evaluation of each gizzard. Grading scales were determined for gross lesions and histopathology upon the assessment of trends seen in the data.

INTRODUCTION

The core of the broiler industry is surrounded by multiple aspects that allow for the most efficient growth of birds prior to harvest. Considerations of genetics, parent flock health, brooding practices, starting chicks, diet and management throughout the life of each flock before a harvest are closely monitored and constantly improved upon. With the demand placed on broiler growth, gastrointestinal health is imperative for a successful flock harvest. The gizzard is the major part of the gastrointestinal control of feed particle size before absorption occurs. The gizzard is a thick muscular organ that is able to finely grind the feed a bird intakes. With the demand on reducing a diet into fine particles the gizzard muscles become "stronger" with bright red muscle appearance and the koilin is normally thick and abrasive with most commercial poultry diets. There have been extensive reports in broiler chickens of gizzard and koilin erosions. Etiologies have linked gizzard erosions with feed particle sizes, nutritional deficiencies, toxic compounds in the feed, microbial etiologies, viral etiologies and even congenital causes from broiler breeder's feedstuff (Gerd Gjevre).

MATERIALS AND METHODS

Birds. A total of 100 broiler chickens were allocated for this study and chosen in a randomized method at the intervals of 1, 14, 28, 42, 49, and 56 days of age from two flocks: No antibiotics ever(NAE) and conventionally raised. The birds were housed, managed and fed according to the corporate polices of a commercial Alabama broiler company.

Collection. Ten birds were randomly chosen from each broiler farm and presented to the PRDL for necropsy and sample collection. Allometrics of body weight (BW), gizzard weight (GW), gizzard length (GL), proventricular length (PL), BW:GW and GL:PL were measured.

Gross gizzard scores were determined (Grade 0=no koilin erosion, no mucosal involvement; Grade 1=superficial koilin erosion, no mucosal involvement; Grade 2=partial thickness koilin erosion, no mucosal involvement; Grade 3=full thickness koilin erosion, and musosal involvement). Full or partial gizzards from each bird were collected and fixed in 10% neutral buffered formalin. Gizzards from each of (36/100) gross scoring categories, collated at each age interval, ranging from no lesion to severe erosion were processed and individual gizzards were histologically evaluated and scored for multiple categories (adhered koilin surface bacteria, koilin inflammatory entrapment, koilin or submucosal hemorrhages, koilin attachment to glands, glandular inflammation, and submucosal inflammation).

RESULTS

NAE Birds. Average body weights: 1d(4.173g), 14d(401.32g), 28d(1358.56g), 42d(2823g), 56d(4048.5g). Average gizzard weights: 1d(1.946g), 14d(10.667g), 28d(8.2g), 42d(10.86g), 56d(40.95g). Average gizzard lengths:1d (1.82cm), 14d(3.87cm), 28d(5.08cm), 42d(6.68cm), 56d(6.2cm). Gross score

average: 1d(1.4), 14d(1.2), 28d(1.8), 42d(1.5), 56d(1.0). Average koilin thickness: 1045.731µm. Average glandular thickness: 895.845µm.

Conventional Birds. Average body weights:1d(45.526g), 14d(431.13g), 28d(1347.461g), 42d(2819g),, 49d(7113g). Average gizzard weights: 1d(2.892g), 14d(14.32g), 28d(27.288g), 42d(40.5g), 49d(36.66g). Average gizzard lengths: 1d(2.01cm), 14d(2.49cm), 28d(3.76cm), 42d(3.77cm), 49d(4.8cm). Gross score average: 1d(1.2), 14d(1.1), 28d(1.3), 42d(0.6), 49d(1.1). Average koilin thickness: 658.918µm. Average glandular thickness: 765.282µm.

DISCUSSION

The gizzard is an important part of ensuring broilers are able to properly digest and convert feed into a sellable meat product. With current demands being put upon the poultry industry to raise broilers without the use of antibiotics additional understanding of any changes in normal physiology must be addressed in order to ensure appropriate broiler growth. Changes of the gizzards observed in this study revealed more severe gizzard erosions in birds raised with no antibiotic ever verses the conventionally raised birds. This study is considered an initial evaluation to determine gross and histological means to evaluate gizzard erosions in broiler chickens raised without antibiotics. Additional exploration of etiologies leading to the changes in the NAE gizzards is still under assessment and further exploration is warranted.

USING BLACK SOLDIER FLY LARVAE TO SUPPLEMENT METHIONINE LEVELS IN ORGANIC LAYER RATIONS

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ABSTRACT

Adequate methionine levels in poultry have been associated with proper development, improved egg production and overall improvement in bird health. In the United States,' the USDA's National Organic Program (NOP) organic regulations limit the use of supplemental synthetic methionine in poultry raised organically. There are even indications that synthetic methionine may be phased out altogether in the future in organic rations. This restriction of synthetic methionine supplementation generates a need for a viable organic methionine supplement that is cost-effective for farmers and does not negatively affect the health and welfare of the birds or the taste and quality of the eggs. In order to address this, black soldier fly larvae were added to layer rations at (w/w) concentrations of 20%, 15%, and 10%. Specifically, organic layers were fed either a standard layer ration with no added methionine or the same standard layer ration with added BSFL from two commercial sources. Welfare, egg quality and taste were evaluated. Results showed a significant increase in the prevalence of footpad dermatitis in birds fed 10% BSFL relative to the control diet as well as a significant decrease in yolk color in birds fed 15% and 10% BSFL relative to the control diet. Aside from those differences there were no other significant differences between the BSFL raised birds and the control birds with respect to welfare, egg quality and taste. While insects are currently not FDA approved as a food additive for livestock and poultry, BSFL are a promising supplement with respect to addressing methionine deficiencies in organic layer diets that also fit the natural nutritional biology of birds foraging behaviors.

INTRODUCTION

Methionine is an essential and first limiting amino acid for poultry and is associated with proper development, improved egg production, feed conversion, and overall health (9). In chickens, methionine deficiency can cause a variety of problems from both a welfare and a production standpoint. In terms of the hens' welfare, methionine deficiency has been shown to encourage feather picking and cannibalism (12), which can subsequently contribute to poor productivity. A methionine deficiency can also decrease the birds' egg production, egg weights, and egg mass (4), affecting the profit margins of the farmer.

In United States organic poultry production, the United States Department of Agriculture (USDA) limits the quantity of synthetic methionine farmers can add to their chicken feed (5). There are even indications that this synthetic supplement may be phased out altogether in the future, generating a need for a viable organic alternative (5). Specifically, layer hens require approximately 0.3% to 0.36% methionine in their diets, depending on the breed (5). Lohman Brown layers require 0.36% methionine when they are laying (7). Alternatives for methionine supplementation such as fishmeal have been explored, but due to the diminishing supply and the elevated costs associated with that, as well as its negative effects on the sensory qualities of eggs (3), a more cost-effective, easily produced alternative that does not negatively affect the taste or quality of the eggs is needed.

Previous efforts have identified black soldier fly larvae (BSFL) as a potentially viable organic source of methionine for layer hens due to its nutritional composition, ease of growth, and cost-effectiveness (2). BSFL has been fed to broilers as a substitute for soymeal, and the chicks were found to have the same weight gain as the soymeal raised chicks with less feed consumed, indicating an improved feed conversion ratio (8). Black soldier flies have also been fed successfully to rainbow trout as a protein source replacement without affecting the sensory qualities of the meat (10). Nutritionally, BSFL contain 40-44% crude protein, with 1.9-2.5% of the crude protein

containing methionine, although the exact nutrient composition of the insect is dependent on the substrate it is grown on (8). BSFL are able to grow from a wide variety of substrates, including food waste and manure, and have a high feed conversion efficiency (8). If used for organic poultry production, however, the substrates they can be raised on are restricted to organic sources. Due to its high dry matter content, BSFL is also easy and cheap to dehydrate and grind into meal (8).

We hypothesize that laying hens supplemented with BSFL as a methionine supplement will have no differences in welfare, egg quality and sensory evaluations when compared to groups fed the same standard layer ration with no added synthetic methionine.

MATERIALS AND METHODS

Birds and management. The experiment was carried out between September and October 2017, during the time when the layers were 21 to 26 weeks of age. 181 organic, organic-raised Lohman-Brown layer hens were randomly divided into four groups: two control flocks fed a standard layer mash ration with no additional methionine supplement (n=46 and 45), and two experimental flocks fed the same standard layer ration with ground BSFL as a methionine supplement (n=45 and 45). Experimental flock 1 was given larvae sourced from Tastyworm (0.59% Methionine content) while experimental flock 2 was given larvae sourced from Naturespeck (0.78% Methionine content). Each group of hens was housed in an identical 10'x10' coop and provided approximately 33'x33' of fenced-in pasture space seeded with GoundWork® All-Purpose Forage. Hens were given ad libitum access to food and water. During the experiment, the coops were not moved, so pasture was present until fully eaten.

Over the course of the six weeks that data was collected, the percentage by weight of BSFL in the experimental flocks' diets was altered every other week: 20% during weeks 21-22, then 15% during weeks 23-24, and finally, 10% during weeks 25-26. The first week of each new diet was considered a "wash week", where no egg data was collected as to allow for any changes in the diet to fully show up in the eggs.

Animal welfare and health. Animal welfare assessments were performed by randomly selecting twelve birds from each group and assessing their walking ability, footpad condition, and feather condition. These assessments were conducted eight times: once a week from weeks 20 to 27. Lameness, limping, lethargy, and hesitation to walk were recorded as present or not present during walking assessments. Footpad conditions were scored on a 0 to 4-point scale: a score of 0 indicated no prevalence of footpad dermatitis while a score of 1, 2, 3, and 4 indicated that less than 10%, 10-25%, 25-50%, and over 50% of the footpad was affected by footpad dermatitis respectively. Feather conditions were scored on a 0 to 3-point scale: a score of 0 indicated no feather loss; a score of 1 indicated feather loss in one localized area; a score of 2 indicated minor feather loss in multiple areas; and a score of 3 indicated severe feather loss.

Mortality data were collected throughout the experiment.

Sensory evaluations. Eggs for sensory evaluations were collected on weeks 22, 24, 26, and 29. They were hard boiled, peeled, cut, and plated for evaluation. Eggs that were collected on weeks 22 (20% BSFL), 24 (15% BSFL), and 26 (10% BSFL) were presented in three pairs: control Group 1 versus control Group 2, experimental Group 1 versus a control, and experimental Group 2 versus a control. Eggs collected on week 29 were only collected from control Group 2. Different slices from the same egg were presented as a pair, and each tester was again presented three pairs of eggs. Testers were asked to record whether or not they tasted a difference between each pair of eggs, and if so, what that difference was.

Egg quality. Twenty eggs were collected from control Group 1, experimental Group 1, and experimental Group 2 for quality analysis on weeks 22 (20% BSFL), 24 (15% BSFL), and 26 (10% BSFL). They were then stored at 4oC for 19 hours then analyzed for egg weight, egg volume, shell thickness, yolk color, and albumen height. Haugh units and specific gravity were then calculated for each egg.

Yolk color was measured using a Roche Yolk Colour Fan, which grades the color of an egg from 1 (lightest) to 15 (darkest). Due to the subjectivity of yolk color measurements, two personnel were instructed to take measurements independently, then the average of these measurements was recorded. The albumen height was measured using an albumen height gauge at three different locations on the egg to the nearest tenth of a mm. The average of those measurements was recorded. The specific gravity of each egg was then calculated by dividing the weight (g) of the egg by the volume (mL) of the egg. The Haugh unit, a measure of egg protein quality based on albumen height, for each egg was also calculated afterwards using the formula: Haugh unit= 100*log_10⁡〖(7.6+ Albumen Height (mm)-1.7*〖Weight(g)〗^0.37)〗.

Statistical analysis. Descriptive statistics and parametric statistics were done on R v3.4.3.

RESULTS

Animal welfare and health. No problems with walking ability were observed in any of the four groups during the assessment period. Feather scores of 0 were also observed throughout all four groups during the entirety of the trial. There was a significant difference between the proportion of control birds versus both experimental group birds presenting with footpad dermatitis (foot score > 0) during weeks 25-26, during the 10% BSFL trial. Both experimental groups demonstrated a greater prevalence of footpad dermatitis ($p=0.0243$) than both control groups.

Three birds were euthanized from control Group 2 after demonstrating signs of lethargy and unresponsiveness. Necropsy results indicated that the birds passed from proventricular/ventricular and duodenal grass impactions. One bird was euthanized from control Group 3 for lethargy and labored breathing, but necropsy and pathology results showed no sign of abnormalities.

Sensory evaluations. After elimination of tasters who identified a difference between the two controls, differences between pairs of eggs from different groups were compiled (Table 1). The proportions of tasters that perceived a difference between control groups and experimental groups during the three BSFL trials (20%, 15%, and 10%) ranged from 43% to 72%. Despite these high proportions, when comparing these to the proportions of tasters that perceived a difference between a pair of control eggs or between two slices of the same control 2 egg, results showed no significant difference. The exception to this was the proportion of perceived differences between control eggs and 15% BSFL experimental two eggs, which was significantly ($P=0.0215$) lower than the proportion of perceived differences between control 1 and control 2 eggs, but not significantly different than the proportion of differences between two slices of the same egg.

Egg quality. When comparing eggs collected during the same week, no significant difference ($P<0.05$) was found between the egg weights, shell thicknesses, albumen heights, Haugh units, or specific gravities of control Group 1, experimental Group 1, and experimental Group 2. However, eggs from both experimental groups were both found to have significantly lighter yolk colors than control Group 1 eggs during the 15% BSFL trial ($P<0.01$), and eggs from experimental Group 2 were found to have significantly lower yolk color than the control Group 1 eggs during the 10% BSFL trial ($P<0.05$).

DISCUSSION

Animal welfare and health. Although a greater percentage of birds with foot pad dermatitis in the experimental groups was observed, no biological explanation could be found in the literature to support this finding. In contrast, no difference in the feather condition scores was noted, which has been correlated with methionine levels in the feed (12). In summary our results suggest that birds fed BSFL at 20%, 15%, and 10% of their diet by weight did not have any statistically significant negative welfare or health consequences from the novel methionine supplement.

Grass impactions, as observed in control Group 2, has been rated as one of the top potential problems in organic and free-ranged poultry production (11). It is possible that a deficiency in methionine contributed to the increased grass consumption of these birds as they consume to meet the energy requirement for the amount of eggs it is producing, which is controlled in part by the amount of methionine they receive (6). This would suggest that the birds with added BSFL were given an adequate supplement to prevent these problems. However, due to the variability in individual bird consumption of forage, it is not possible to say conclusively whether this was a significant factor.

Sensory evaluations. Since no significant difference was found in the proportions between testers who reported a difference between two slices of the same egg and the proportions of testers who reported a difference between the control and experimental eggs, we can conclude that there was likely no actual difference in the sensory qualities of the eggs.

Egg quality. The only significant difference observed in egg quality between the control and experimental groups was yolk color during 15% and 10% BSFL trials (Table 2). These results are consistent with a previous study by Al-Qazzaz (1), where hens given greater amounts of insect meal produce eggs with lighter yolks. Interestingly, no significant color changes were noted during the 20% BSFL trial. Yolk color is primarily derived from carotenoids in their diet, so it is possible that during the beginning of the study, when the experimental group were fed 20% BSFL, the pasture was denser, which may have provided more carotenoids to the birds and contributed to the decrease in yolk color score.

This study suggests that BSFL can be an effective organic supplement of methionine for layers. However, current FDA regulations would need to be amended as they prohibit the use of insect meal in organic poultry feed (2). Adding the larvae as a methionine supplement to feed has not shown any significant detrimental effect on the health or welfare

of the birds, nor on the taste or quality of the eggs produced. Fatty acid analysis of the eggs from all four groups is still being conducted to determine if the egg nutrient contents have varied from the change in diet. Future studies will aim to look at a similar experiment with broilers and include production data (uniformity, feed conversion ratio, body weight, etc) that was not collected for each experimental group during this study, as well as compare BSFL diets to a standard ration with added methionine.

REFERENCES

1. Al-Qazzaz, M. F. A., D. Ismail, H. Akit, and L. H. Idris. Effect of using insect larvae meal as a complete protein source on quality and productivity characteristics of laying hens. Revista Brasileira de Zootecnia 45:518-523. 2016.

2. Burley, H., P. Patterson, and K. Anderson. Alternative ingredients for providing adequate methionine in organic poultry diets in the United States with limited synthetic amino acid use. World's Poultry Science Journal 71:493-504. 2015.

3. Elswyk, M., P. Dawson, and A. Sams. Dietary menhaden oil influences sensory characteristics and headspace volatiles of shell eggs. Journal of Food Science 60:85-89. 1995.

4. Elwinger, K., M. Tufvesson, G. Lagerkvist, and R. Tauson. Feeding layers of different genotypes in organic feed environments. British Poultry Science 49:654-665. 2008.

5. Fanatico, A. Organic poultry production: providing adequate methionine. Citeseer. 2010.

6. Harms, R., G. Russell, H. Harlow, and F. Ivey. The influence of methionine on commercial laying hens. Journal of Applied Poultry Research 7:45-52. 1998.

7. Lohmann, H. Management Guide for Lohmann Brown-Classic. Lohmann Tierzucht GmbH, Cuxhaven, Germany. 2010.

8. Makkar, H. P., G. Tran, V. Heuzé, and P. Ankers. State-of-the-art on use of insects as animal feed. Animal Feed Science and Technology 197:1-33. 2014.

9. Schutte, J., J. De Jong, and H.-L. BERTRAM. Requirement of the laying hen for sulfur amino acids. Poultry Science 73:274-280. 1994.

10. Sealey, W. M., T. G. Gaylord, F. T. Barrows, J. K. Tomberlin, M. A. McGuire, C. Ross, and S. St-Hilaire. Sensory analysis of rainbow trout, Oncorhynchus mykiss, fed enriched black soldier fly prepupae, Hermetia illucens. Journal of the World Aquaculture Society 42:34-45. 2011.

11. Singh, M., and A. J. Cowieson. Range use and pasture consumption in free-range poultry production. Animal Production Science 53:1202-1208. 2013.

12. Tiller, H. Nutrition and animal welfare in egg production systems. In: Proceedings of 13th European Symposium on Poultry Nutrition. pp 226-232. 2001.

Table 1. Proportion of testers who reported a difference in the sensory evaluation of two groups of eggs.

Eggs compared	Proportion Different
Control vs 20% experimental 1[A]	65% (n=31)
Control vs 20% experimental 2 [A]	59% (n=29)
Control vs 15% experimental 1 [A]	71% (n=41)
Control vs 15% experimental 2 [A]	43% (n=40)[B]
Control vs 10% experimental 1 [A]	63% (n=43)
Control vs 10% experimental 2 [A]	72% (n=43)
Control 1 vs Control 2	62% (n=305)[B]
Control 2 vs Control 2	56% (n=272)

[A] Testers who claimed to taste a difference between the two control groups were not included

[B] P=0.021

Table 2. Egg quality analysis averages during each BSFL trial.

Group	BSFL (age)	Egg Weight (g)	Shell thickness (mm)	Yolk Color (Roche score)	Albumen Height (mm)	Haugh Units	Specific Gravity (g/mL)
Control group 1	--- (22wk)	51.36	0.35	13.10	8.33	92.94	1.09
Experimental group 1	20% (22wk)	52.06	0.36	12.74	9.31	97.66	1.10
Experimental group 2	20% (22wk)	51.81	0.36	12.20	8.53	94.21	1.110
Control group 1	--- (24wk)	52.18	0.36	12.18^{AB}	7.90	91.12	1.08
Experimental group 1	15% (24wk)	53.18	0.36	10.90^{A}	8.40	93.19	1.099
Experimental group 2	15% (24wk)	53.54	0.34	10.84^{B}	8.64	91.38	1.09
Control group 1	--- (26wk)	54.15	0.35	13.31^{C}	8.11	91.48	1.10
Experimental group 1	10% (26wk)	56.52	0.35	12.89	8.4	92.24	1.11
Experimental group 2	10% (26wk)	53.09	0.33	12.39^{C}	8.24	92.19	1.08

$^{A}P=0.007$, $^{B}P=0.003$, $^{C}P=0.046$

ASSESSMENT AND MITIGATION OF CONTAMINATION RISKS: CRITICAL KNOWLEDGE TO REDUCE DISEASES AND INCREASE BIOSECURITY COMPLIANCE

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INTRODUCTION

The link between infectious disease transmission and mechanical vectors (ex: boots, vehicles) has been demonstrated in numerous studies (1, 2, 3) and led to the creation of biosecurity protocols. However, few studies have quantified compliance (4, 5) and for those who have estimated it, low compliance with biosecurity measures when entering and exiting barns has been demonstrated in poultry and swine farms particularly, all around the world. On poultry farms in Québec, overall compliance of barn entrance biosecurity protocols was only 25.7% to 34.7% in 2012 based on videosurveillance (6). Changing boots and respecting the different barn entrance areas had a compliance of 46.1-52.3% and 13.3-17.9% respectively. As stated above, Québec is no different to what has been observed elsewhere; which is an important issue since, to be effective, biosecurity measures must be consistently applied. A lot of training material has been developed to help increase biosecurity compliance. But, a fundamental component of educational products designed for adult learning is information explaining why what is taught is important. This research is assessing the contamination caused by the no respect of clean and dirty areas at barn entrances, since it was the most significant problem highlighted by Racicot et al. in 2011. Our goal is to visually show, using real pathogens, how they spread so that we can quantify the impact of biosecurity breaches and obtain in the process valuable training material.

MATERIALS AND METHODS

Floor and boot contaminations were evaluated following three biosecurity breaches, which have been identified as most frequent in a study by Racicot et al (2011):

1. Donning farm boots while in the clean area
2. Donning farm boots while in the dirty area (instead of donning them while crossing the delimitation (hygiene barrier) between the dirty and the clean areas)
3. Not changing boots between the dirty and the clean areas

These breaches were performed using used farm boots (clean and disinfected beforehand) taken from a poultry farm near the Faculty of Veterinary Medicine of the Université de Montréal. The second breach (donning farm boots while in the dirty area) was also performed using disposable plastic boots. Afterward, floor and boot contaminations of these three breaches were compared to the control scenario which is an industry-recommended controlled procedure i.e. donning farm boots while crossing the delimitation (hygiene barrier) between the dirty and the clean areas. Here are the different scenarios performed during the project:

Control: Changing boots while crossing the delimitation (hygiene barrier) between the dirty and the clean areas (Control Scenario; C)

Donning farm boots while in the clean area (Scenario 1; S1)

Donning farm boots while in the dirty area

- With rubber boots (Scenario 2; S2)
- With plastic boots (Scenario 3; S3)

Not changing boots between dirty and clean areas (Scenario 4; S4)

Two organisms were tested independently for each scenario; a generic *E. coli* Ampicillin-resistant strain and a T4 bacteriophage (acting as a surrogate for viral pathogens).

Statistical analysis. All scenarios were performed eight times (8 times x 2 pathogens x 5 scenarios) for a total of 80 tests. Furthermore, for Scenario 4 bacteria tests, we took floor samples of the heel and the toe. Plaque-forming Units (bacteriophage) and Mean Greyness (bacteria) were transformed using the logarithm base 10 to normalize the distributions. Mixed linear models using SAS v. 9.3 (Cary, N.C.) were performed to compare scenario values with an alpha value set at 5%. A priori contrasts were used to compare pairs of means adjusting the

alpha level with the sequential Benjamini-Hochberg method.

RESULTS & DISCUSSION

1. Inter-Scenarios Analysis

Floor contamination with bacteriophage

There was no statistical difference between the left and right foot ($p = 0.12$) (no foot difference) and this was the case for all scenarios ($p=0.83$) when comparing all the steps for the different scenarios (C, S1, S2, S3 and S4), i.e., there was no foot difference in general ($p = 0.24$), no interaction between the foot (right or left) and the scenario ($p = 0.80$) and between the foot and the step number (each step from the dirty area to the clean area were sequentially numbered with the highest number being the farthest away from the clean area; $p = 0.17$). For that reason, the contrasts are reported with no distinction between left or right foot.

When comparing the first step of the control scenario to scenarios S1, S2, S3 and S4, there was no statistical difference ($p > 0,05$ or non-significant after adjustment). Same result came when comparing the first step between the biosecurity breach scenarios S1, S2, S3 and S4 ($p > 0,05$ or non-significant after adjustment).

For the second step, S1 and S4 were significantly higher than S2 and S3 ($p < 0.0001$). No difference between S1 and S4 ($p=0.87$).

The third step was skipped to reduce research costs.

For the fourt, fifth and sixth steps, S4 (not changing boots) was significantly higher than S1, S2 and S3 ($p < 0.0001$).

Floor contamination with bacteria

The positive control for S4 was significantly lower than S2 ($p = 0.0001$) and S3 ($p = 0.0008$). All positive controls were between 3.29 and 3.97 log. All negative controls for the floor in the Outside area from all scenarios were not statistically different ($p = 0.27$): no foot difference ($p = 0.34$) and no interaction between the foot (right or left) and the scenario ($p = 0.39$). All negative controls for the sterile organic material were also not significantly different between scenarios ($p = 0.10$).

At the first step, S2 was significantly higher than the control scenario ($p < 0.0001$), S1 ($p < 0.0001$) and S4 ($p < 0.0001$). S3 was significantly higher than S4 ($p = 0.0001$).

At the second step, the control scenario was significantly lower than S1 ($p < 0.0001$), S2 ($p = 0.0001$) and S4 ($p < 0.0001$). S3 was significantly lower than S1 ($p < 0.0001$) and S4 ($p < 0.0001$).

At the third step, the control scenario was significantly lower than S2 ($p = 0.001$) and S4 ($p < 0.0001$). S4 was significantly higher than S1 ($p < 0.0001$) and S3 ($p < 0.0001$).

At the fourth step, the control scenario was significantly lower than S4 ($p < 0.0001$). S4 was significantly higher than S1 ($p < 0.0001$) and S3 ($p < 0.0001$).

At the fifth step, the control scenario was significantly lower than S4 ($p < 0.0001$). S4 was significantly higher than S1 ($p = 0.0005$) and S3 ($p = 0.0002$).

At the sixth step, the control scenario was significantly lower than S4 ($p < 0.0001$). S4 was significantly higher than S1 ($p < 0.0001$) and S3 ($p < 0.0001$).

Boot contamination with bacteriophage

The mean phage contamination of the dirty area boots of S3 was significantly lower than S2 ($p=0.015$), with a difference of 0.34 log. This difference is marginal compared to the difference observed between dirty and clean area boots contaminations (3.07-4.44, log difference) in scenario control, S1, S2 and S3. For clean area boots, if we exclude the control scenario (since all values are below detection limits), no statistical difference was recorded between the different biosecurity breach scenarios, except for S4 (no change of boots) that had a contamination level significantly higher than the other scenarios ($p<0.05$) There is between S4 clean area boots and the other errors scenarios S1, S2 and S3, a log difference of 3.73, 2.80 and 2.72 respectively. The clean area boot contamination of scenarios S1, S2, S3 and S4 were higher than the control boots by 0.28, 1.21, 1.9 and 4.01 log respectively. An interesting fact is that the average contamination of S4 boots is no different from the average contamination of the dirty area boots for all the other scenarios (C, S1, S2 and S3), which mean that walking over 10m doesn't reduce boots contamination.We also looked at the difference between dirty and clean areas boots of S1, S2, and S3 (excluding S4 because there was no change of boots; and the control scenario, because there was no variance with all results being below the detection limit). The contamination level of the clean area boots was significantly lower than the dirty area boots ($p < 0.0001$) for all scenarios. So there is no scenario effect on the contamination level of the S1, S2, and S3 clean area boots.

Boot contamination with bacteria

All negative controls of the dirty area boots from all scenarios were not statistically different ($p = 0.35$): no boot difference ($p = 0.82$) and no interaction between the foot (right or left) and the scenario ($p = 0.09$). There was no statistical difference between the dirty area boots of the control scenario, S1 and S2: no foot difference or scenario effect ($p > 0.05$). For the control scenario, the dirty area boots were

significantly more contaminated than negative controls ($p < 0.0001$); but the contamination of the clean area boots was not statistically different than the negative controls (for toe, $p= 0.79$ and heel samples, $p=0.97$). However, for S1, S2, S3 and S4, all boots were statistically more contaminated than negative controls ($p < 0.05$). This means that all errors scenarios create contamination on the clean area boots. When comparing the dirty and clean area boots (Control, S1 and S2), the contamination significantly decreased from the former to the latter in all of these three scenarios. This means that changing boots reduce contamination level on clean area boots but does not prevent contamination. When comparing the heel and toes of the clean area boots (Control, S1, S2, S3 and S4), there was no statistical difference between their contamination levels for a given scenario ($p > 0.05$ or not significant after adjustment). For toes, the control and S1 scenarios were significantly less contaminated than for S2 and S4 ($p < 0.05$). We hypothesize that maybe the presence of sterile organic material on the floor of the dirty area (S2 and S4) may increase boot contamination on the toe region compare to when boots are changed in the clean area or over the red line. Toe contamination for S3 was significantly lower than for S2 ($p=0.0019$). It may be that plastic boots keep less contaminant material than rubber boots (S3 vs S2). For heels samples, the control scenario was significantly lower than S2 and S4 ($p< 0.0001$), and S1 was significantly lower than S4 only ($p =0.0004$) (compared to S1 that had a significantly lower contamination level than S4 and S2).

2. Individual scenario analysis

Control scenario

Floor contamination with bacteriophage: The mean contamination at the first step was 5.78 logs and all remaining steps were below detection limit (i.e. 2 logs), so no statistical analysis was possible since there was no variance except for the 1st step.

Floor contamination with bacteria: Negative controls had a significantly lower contamination level than the first step (dirty area; $p < 0.0001$), but there was no statistical difference when compared to step two ($p = 0.75$), 3 ($p = 0.66$), 4 ($p = 0.99$), 5 ($p = 0.92$) and 6 ($p = 0.80$) (steps in the clean area). The first step contamination was significantly higher than all the other steps ($p < 0.0001$).

Boot contamination with bacteriophage: The phage contamination of the dirty area boots had a mean of 6.44 logs and the clean area boots had a contamination level always below the detection limit (2 logs). Since there was no variance for the results obtained from the clean area boots, no statistical analysis was possible. There is a mean of 4.44 log difference between the dirty area and the clean area boots (or at least a 4.15 log difference).

Boot contamination with bacteria: When comparing the right heel of the dirty area boots to the clean area boots, the contamination of the dirty area boots was significantly higher than the clean area boots ($p<0.0001$). Contamination of the dirty area boots was higher than all their negative controls ($p < 0.0001$), but there was no difference between the clean area boot samples and their negative controls (toe $p=0.79$ and heel $p=0.97$).

Scenario 1: Changing boots in the clean area

Floor contamination with bacteriophage: The 4th, 5th and 6th steps were significantly ($p < 0.0001$) less contaminated then 1st and 2nd step ($p = 0.84$).

Floor contamination with bacteria: Negative controls were significantly less contaminated than floor samples of the first ($p < 0.0001$) and second steps ($p < 0.0001$), but no statistical difference was found with step 3 ($p = 0.13$), 4 ($p = 0.53$), 5 ($p = 0.26$) and 6 ($p = 0.18$). First step samples were significantly more contaminated than all the other steps ($p < 0.0001$) except for step 2 ($p=0.08$).

Boot contamination with bacteriophage: The mean phage contamination of the clean area boots was significantly lower than the dirty area boots ($p < 0.0001$) with a difference of 3.71logs.

Boot contamination with bacteria: when comparing the right heel of the dirty area boots to the one of the clean area boots, the dirty area boots had a significantly higher contamination than the clean area boots ($p<0,0001$). All samples from the dirty and clean area boots were significantly more contaminated than the negative controls ($p < 0.05$).

Scenario 2: Changing boots in the dirty area with rubber boots

Floor contamination with bacteriophage: The 1st step was significantly higher than the others ($p < 0.0001$)

Floor contamination with bacteria: Negative controls were significantly less contaminated than samples for all steps: step 1 ($p < 0.0001$), step 2 ($p < 0.0001$), step 3 ($p < 0.0001$), 4 ($p = 0.0002$), 5 ($p = 0.0009$) and 6 ($p = 0.001$). First step contamination was significantly higher than all the other steps ($p < 0.0001$).

Boot contamination with bacteriophage: The mean phage contamination of the clean area boots was significantly lower than the dirty area boots ($p < 0.0001$) with a difference of 3.49 logs.

Boot contamination with bacteria: When comparing the right heel of the dirty area boot to the one of the clean area boot: the dirty area boot was significantly more contaminated than the clean area boot ($p < 0.0001$). All samples from the dirty and clean

area boots were significantly higher than the negative controls ($p < 0.05$).

Scenario 3: Changing boots in the dirty area with plastic boots

Floor contamination with bacteriophage: The 1st step was significantly more contaminated than the others ($p < 0.0001$).

Floor contamination with bacteria: Negative controls were significantly less contaminated than floor samples of the first step ($p < 0.0001$), but there was no statistical difference compared to step 2 ($p = 0.03$; non-significant after adjustment), 3 ($p = 0.53$), 4 ($p = 0.62$), 5 ($p = 0.80$) and 6 ($p = 0.42$). First step boots were significantly more contaminated than for all the other steps ($p < 0.0001$).

Boot contamination with bacteriophage: The phage contamination of the clean area boots was significantly lower than the dirty area boots ($p < 0.0001$) with a difference of 3.08 logs.

Boot contamination with bacteria: When comparing clean area boots to their negative controls, the clean area boots were significantly more contaminated than these negative controls ($p < 0.05$).

Scenario 4: Not changing boots between areas

Floor contamination with bacteriophage: The 1st step was significantly more contaminated than 4th step ($p < 0.0004$), but it was not different from the 2nd ($p=0.45$), 5th ($p=0,.006$, non-significant after adjustment) and 6th steps ($p=0.004$, non-significant after adjustment).

Floor contamination with bacteria: Toe and heel negative controls were significantly less contaminated than for all steps: step one (toe: $p = 0.0005$ and heel: $p = 0.001$), step two ($p < 0.0001$ and $p < 0.0001$), step three ($p < 0.0001$ and $p < 0.0001$), 4 ($p = 0.0004$ and $p = 0.0001$), 5 ($p = 0.0008$ and $p = 0.0008$) and 6 ($p < 0.0001$ and $p < 0.0001$). First step samples were not significantly different than all the other steps.

Boot contamination with bacteriophage: The mean contamination of the boots at the end of the clean area was 6.01 logs and it was not statistically different from the contamination level of the dirty area boots of other scenarios ($p > 0.41$).

Boot contamination with bacteria: When comparing boot samples, taken at the end of the clean area, to the negative controls, these were significantly higher than the negative controls ($p < 0.05$).

MAIN CONCLUSIONS

- Preventing cross-contamination of areas with boot changing is an effective way of preventing the spread of contamination
- Floor and boots are contaminated by all the most common types of breaches observed on Canadian farms
- Floor and boot contaminations are higher when boots are not changed compared to when boots are inappropriately changed
- When boots are not changed, there is no dilution effect of the contamination by walking over a 10 m distance.

REFERENCES

1. Vaillancourt, J-P., A. Martinez, and al. (2000). The epidemiology of Mycoplasma gallisepticum in North Carolina. 34–36.
2. Fasina, F. O., et al. (2011). Identification of risk factors associated with highly pathogenic avian influenza H5N1 virus infection in poultry farms, in Nigeria during the epidemic of 2006–2007. 98(2): 204-208.
3. Nishiguchi, A., et al. (2007). Risk Factors for the Introduction of Avian Influenza Virus into Commercial Layer Chicken Farms During the Outbreaks Caused by a Low-Pathogenic H5N2 Virus in Japan in 2005. 54(9-10): 337-343.
4. Vaillancourt J.-P., Carver D.K. (1998). Biosecurity: perception is not reality. 57(6), 28-36.
5. Racicot M., Venne D., Durivage A. and J.P. Vaillancourt (2011). Description of 44 biosecurity errors while entering and exiting poultry barns based on video surveillance in Quebec, Canada. 100 (3-4): 193-199.
6. Racicot M., Venne D., Durivage A. and J.P. Vaillancourt (2012). Evaluation of strategies to enhance biosecurity compliance on poultry farms in Quebec: effect of audits and cameras. 103 (2-3): 208-218.

Figure 1. Layout of the design for each run. Negative controls were taken beforehand in each sampling areas (floor and boots); C+ is the positive control; then 1 to 6 are the different steps. The floor was tested under each step (1 to 6). NOTE: boots were changed between the dirty and the clean areas (except for scenario 4 where the boots were not changed); so, the boots used in the dirty area are named "dirty area boots" and the boots used in the clean area are named "clean area boots."

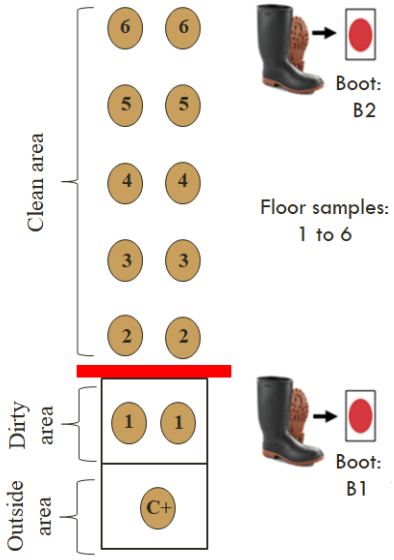
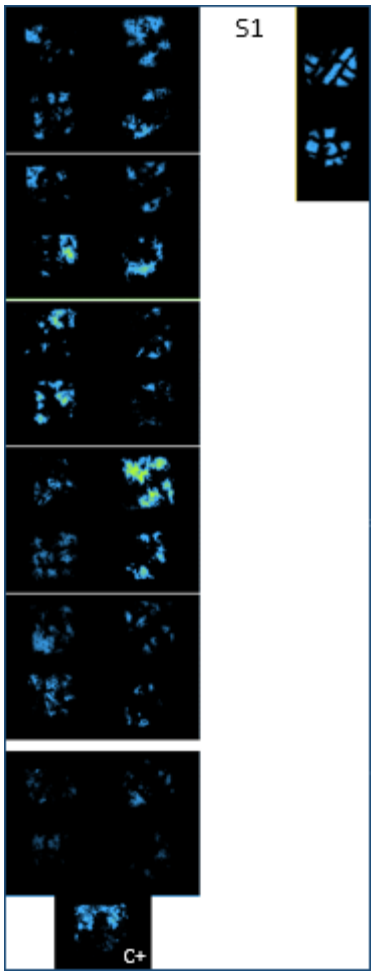
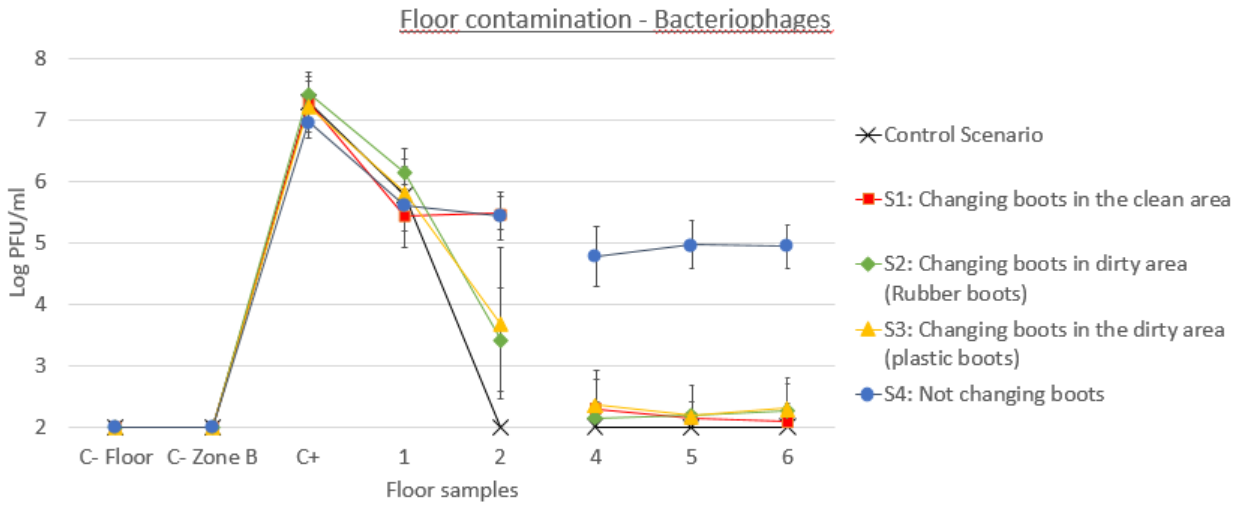


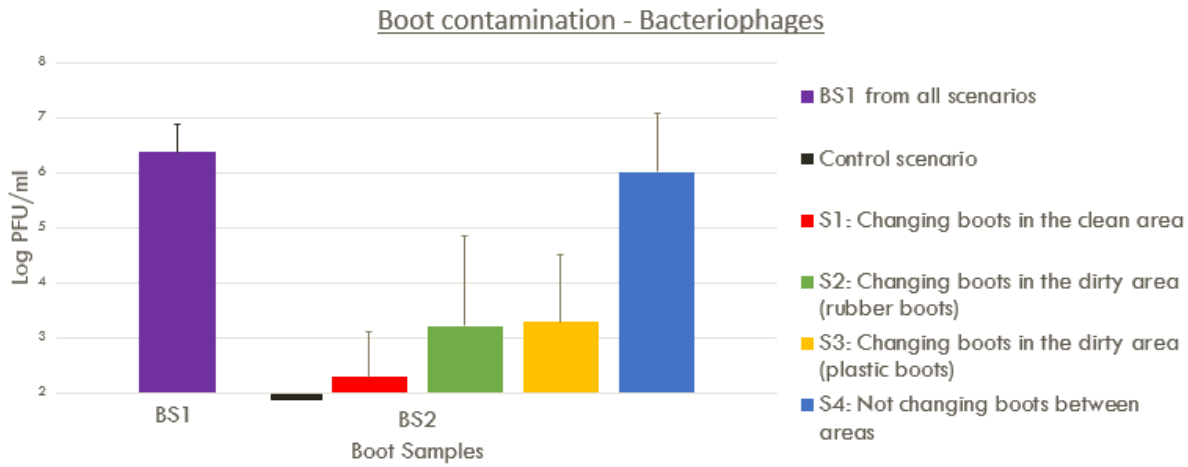
Figure 2. Scenario 4 – Not changing boots. The left side of the figure represent the floor contamination (toe and heel). The right top side of the figure represent boot contamination of the dirty right boot. No dilution effect (reduction of floor contamination) after 10 steps. The underside of the boot is still heavily contaminated after 10 steps.



Graph 1. At the first step (1), all scenarios are similar. At the second step (after the hygiene barrier), S1 and S4 are significantly higher than S2 and S3. At step 4, 5 and 6, S4 is significantly higher than all the other errors scenarios. Floor contamination is higher when boots are not changed compared to when boots are inappropriately changed. Changing boots while crossing the hygiene barrier (Control scenario) is the only scenario preventing floor contamination.



Graph 2. There is no statistical difference between the first pair of boots (BS1) of all scenarios. Changing boots while crossing the hygiene barrier (Control scenario) prevent contamination of the second pair of boots (BS2). For the second pair of boots, there is a statistical reduction in 3 errors scenarios compare to initial contamination (S1, S2 and S3). Not changing boots (S4) significantly increase their contamination by 3-4 log compare to changing boots the wrong way.



VARIABILITY OF *PASTEURELLA MULTOCIDA* STRAINS FROM POULTRY IN ARGENTINA

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ABSTRACT

Overall, 101 *Pasteurella multocida* strains were studied. Eighty-eight strains were mainly isolated from broiler breeder flocks suffering from fowl cholera outbreaks; the remaining 13 strains were isolated from cattle, sheep, swine and rabbits and were included only for comparison purposes. Seventy-five isolates were identified as *Pasteurella multocida* subsp. *multocida*, 25 as *P. multocida* subsp. *gallicida* and one remained unclassified. Using PCR, 80 strains from poultry were allocated as capsular type A, one as F, and seven remained untyped. All strains were examined for their susceptibility to 13 antibiotics, which resulted in 45 different patterns that were grouped into nine clusters; all strains were susceptible to florfenicol; most strains were susceptible to chloramphenicol, trimethoprim plus sulfamethoxazole or tetracycline; more than 80% of the strains were resistant to neomycin, gentamicin or streptomycin while susceptibility to ampicillin, colistin, enrofloxacin, and kanamycin was variable. Furthermore, five virulence genes were sought; detection of genes *pfhA*, *hgbB*, and *nanH* varied among strains whereas none of the poultry strains poultry carried the genes *toxA* or *tbpA*. Fingerprinting by ERIC-PCR grouped poultry strains into 78 patterns, which were further gathered into 41 clones or 25 clusters.

The origin of poultry strains could not be related to any grouping. Nevertheless, when strains were isolated from two outbreaks occurring in the same poultry-farm, ERIC-PCR and antibiotic resistance clearly separated the strains from each outbreak. Applying an exhaustive analysis that included all phenotypic and genotypic assays, a great variation among all strains was revealed. Consequently, it was not possible to establish a typical pattern which would be useful to select candidate vaccine strains that can protect against local pathogenic *P. multocida* strains.

INTRODUCTION

Fowl cholera is a cosmopolitan disease that affects all kinds of birds but often occurs in chickens and turkeys reared under intensive farming conditions (7). In Argentina, fowl cholera outbreaks are important in broiler breeders. The disease takes place as an acute fatal disease or most commonly, as a chronic infection. Vaccination should be considered in areas where fowl cholera is prevalent using either available bacterins and/or live vaccines, but immunization should not be substituted for good sanitary practice (7). Although these vaccines may provide some degree of protection, outbreaks in vaccinated flocks are still being reported, probably due to the lack of cross-protection among local isolates and the international recognized poultry somatic serotypes (A:1, A:3 and A:4), probably due to the local evolution of indigenous strains and the rapid adaptation of *P. multocida* to environmental and host changes.

Therefore, it is suggested that novel vaccines should be prepared including local or regional representative strains that should be selected for other common factors such as capsular types or virulence genes and not only according to somatic serotypes.

MATERIALS AND METHODS

***Pasteurella multocida* strains**. A total of 101 strains of *P. multocida* isolated during 1980-2014 in Argentina were studied. These strains were isolated from fowl cholera outbreaks (88 strains) or from other animal species (six strains from cattle, three from sheep, two from swine, and two from rabbits).

Bacteriology. Primary species identification was done by Gram-Hucker's staining, carbohydrate fermentation tests (4,5) (glucose, lactose, mannitol, sucrose, arabinose, sorbitol, trehalose and xylose) and conventional biochemical tests such as catalase, oxidase, and urease activity, motility, indole and hydrogen sulfide production, ONPG test and growth onto MacConkey agar .

Antibiotic resistance. All strains were examined for their susceptibility to antibiotics using the disc diffusion technique (3). All strains were grown overnight at 37°C onto Columbia Blood agar (CBA) added with 7% defibrinated bovine blood. Growth was suspended in PBS (pH 7.2) until reaching 0.5 McFarland turbidity standard. Using a sterile cotton swab, the suspension was plated onto Mueller Hinton agar plates followed by placing 3-4 discs per plate of

the following antibiotics: ampicillin, chloramphenicol, colistin, enrofloxacin, streptomycin, florfenicol, gentamicin, kanamycin, neomycin, tetracycline and trimethoprim plus sulfamethoxazole (TMS). After overnight incubation at 37°C, each strain was categorized as "Susceptible" (S), "Intermediate" (I) or "Resistant" (R).

Capsular typing, virulence genes and ERIC PCR. One colony per strain was cultivated in Luria-Bertani broth and, after overnight incubation at 37°C, the broth was washed and the DNA was extracted by heating (10 min, 99ºC). Firstly, a multiplex PCR for species-specific amplification of the *kmt* gene and capsular typing (13) was carried out in all strains. Afterwards, another multiplex PCR assay was carried out for the detection of 4 virulence genes: dermonecrotoxin – *toxA*; iron acquisition system – *tbpA* and *hgbB*; and type IV fimbriae – *pfhA* (2). Furthermore, another PCR was done to detect Neuraminidase – *nanH* (4). Finally, ERIC-PCR was performed using primers ERIC1R y ERIC2 (1). Strains were considered to be identical and were allocated into the same pattern if they have 100% similarity, to the same clone if they have 90% similarity (1) or to the same cluster if they have 85% similarity (9).

Statistics. Applying the "Bionumerics" 3.5 test (Applied Maths, Kortrijk, Belgium), ERIC-PCR results were analyzed by estimating banding patterns similarity according to Dice coefficient that generated dendograms based on Unweighted Pair Group Method with Arithmatic Mean (UPGMA). The comparison of relations for *P. multocida* susceptibility to antibiotics was done by using UPGMA with the "R" software (10). Finally, using the Gower's General Similarity Coefficient (8) for similarity with the "R" software, an exhaustive comparison of all available data from strains: province, year of isolation, host species, biotype, virulence genes, ERIC-PCR banding pattern, and susceptibility to antibiotics.

RESULTS

All strains were identified as *P. multocida*: non-motile Gram-negative rods, unable to grow onto MacConkey agar; produced catalase, oxidase and indol but did not produce urease and hydrogen sulfide; were negative to ONPG test; fermented glucose, mannitol and sucrose but did not ferment lactose. According to the differences in the fermentation of arabinose, sorbitol, trehalose, and xylose, the strains were further classified: 75 strains were allocated within subsp. *multocida* and 25 strains within subsp. *gallicida*. Biotyping was referred to Fegan *et al.* (5) where the subsp. *multocida* was distributed into four biovars: biovar one (13 strains); biovar two (16 strains); biovar three (44 strains); and biovar four (2 strains). One strain was provisionally allocated into biovar five (undefined subsp.). Furthermore, 22 strains were classified as subspecies *gallicida* biovar seven while two other strains were sorbitol positive variants of biovar seven and were regarded as biovar 8. Another strain was a sucrose negative variant of the newly mentioned biovar eight and consequently was considered to be biovar nine. Biovars six and 10 correspond to subsp. *septica* and *tigris*, which have not been found in the poultry strains.

Eighty *P. multocida* strains from poultry have a capsule type A as well as six strains from bovines, two strains from rabbits and two of the three strains from sheep. The third strain from sheep belonged to the capsule type D as well as the two strains from swine. One strain from poultry belonged to the capsule type F and it was not possible to allocate seven strains from poultry in any known capsular type.

All *P. multocida* strains were susceptible to florfenicol. Most of the strains were susceptible to chloramphenicol, TMS or tetracycline while more than 80% of the strains were resistant to neomycin, gentamicin or streptomycin. Susceptibility to ampicillin, colistin, enrofloxacin, and kanamycin was variable among strains. Taking in account the susceptibility results of strains, a dendrogram was constructed and the strains were grouped into nine clusters. The biggest cluster included 57 strains and four clusters included eight, nine, 10 or 12 strains. One cluster included two strains from bovines. Finally, three strains represented individually three clusters.

It was found that 73, 59, and 79 *P. multocida* strains from poultry were carrying *pfhA*, *hgbB*, and *nanH* genes, respectively. All non-poultry strains carried *nanH* gene. On the other hand, none of the poultry strains was carrying either *toxA* or *tbpA* gene, whereas only one strain from sheep carried *toxA* gene and seven strains from cattle, sheep and rabbit were carrying *tbpA* gene.

Fingerprinting by ERIC-PCR resulted in the presentation of between 7 and 14 bands, sized 200-1000bp. According to the banding patterns, a dendrogram was constructed grouping these strains into 78 patterns. These patterns were further gathered into 41 clones or 25 clusters (1,9).

DISCUSSION

The use of either live vaccines or inactivated bacterins exclusively based on the international somatic serotypes (A:1, A:3 and A:4) is widely used but afford variable protection (7). The present work was performed to obtain information about similarities and differences among *P. multocida* strains isolated from poultry in Argentina, in order to identify suitable

potential cross-protecting strains, which could be candidates for a novel vaccine.

Most poultry strains belong to the subsp. *multocida* commonly biotypes two and three. The subsp. *multocida* is very common in poultry (5,12) whereas the subsp. *gallicida* was barely found and the subsp. septica and *tigris* (11) have not been described in poultry. In this work, three novel variants of the subsp. *gallicida* biotype seven have been described: two strains that fermented sorbitol and sucrose and one strain only sorbitol.

Using PCR, most poultry strains were allocated as capsular type A. The single type F strain has been isolated from a free-range layer flock from a rural school; although capsular type F have been associated with fowl cholera (7,11), in this case, it is most likely that infection of these hens have been originated from an animal species different from poultry, as in this farm biosecurity measures were poor.

Comparison of susceptibility to antibiotics of all strains resulted in 45 different patterns that were grouped into 9 clusters, which had no epidemiological interpretation or meaning. Nevertheless, strains isolated from the same outbreaks were allocated into the same cluster, showing good discriminatory ability for epidemiological comparisons.

Five virulence genes were studied: *pfhA*, *hgbB*, and *nanH* varied among strains while none of the strains that were isolated from poultry carried either *toxA* or *tbpA*. Similar variability was previously reported among *P. multocida* strains from poultry (4,6).

Using ERIC-PCR, no relationship of the patterns/clones/clusters with the origin of the strains could be found. However, when strains were isolated from two outbreaks occurring at the same poultry-farm, with a few years of difference, ERIC-PCR was capable to clearly differentiate the strains from each outbreak. Therefore, ERIC-PCR may be a suitable technique for studying host adaptation of *P. multocida* and epidemiology of fowl cholera (11).

Applying an exhaustive analysis that included all phenotypic and genotypic assays, it was not possible to establish a typical pattern to select candidate representative strains, which would be able to afford cross-protection between different *P. multocida* strains, due to the great variation among them.

REFERENCE

1. Amonsin, A., J. F. X. Wellehan, L. L. Li, J. Laber, and V. Kapur. DNA fingerprinting of *Pasteurella multocida* recovered from avian sources. J. Clin. Microbiol. 40: 3025–3031. 2002.

2. Atashpaz, S., J. Shayegh, and M. S. Hejazi. Rapid virulence typing of *Pasteurella multocida* by multiplex PCR. Res. Vet. Sci. 87: 355–357. 2009.

3. Bauer, A. W., D. M. Perry, and W. M. M. Kirby. Single disc antibiotic sensitivity testing of Staphylococci. Arch. Intern. Med. 104: 208–216. 1959.

4. Ewers, C., A. Lübke-Becker, A. Bethe, S. Kiebling, M. Filter, and L. H. Wieler.Virulence genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status.Vet. Microbiol. 114: 304–317. 2006.

5. Fegan, N., P. J. Blackall, and J. L. Pahoff. Phenotypic characterisation of *Pasteurella multocida* isolates from Australian poultry. Vet. Microbiol. 47: 281–286. 1995.

6. Furian, T. Q. T., K. A. Borges, S. L. S. Rocha, E. E. E. Rodrigues, V. P. do Nascimento, C. T. P. C. Salle, H. H. L. S. Moraes, V. Pinheiro do Nascimento, C. T. P. C. Salle, and H. H. L. S. Moraes. Detection of virulence-associated genes of *Pasteurella multocida* isolated from cases of fowl cholera by multiplex-PCR. Pesqui. Vet. Bras. 33: 177–182. 2013.

7. Glisson, J. R., C. L. Hofacre, and J. P. Christensen. Pasteurellosis and Other Respiratory Bacterial Infections - Fowl Cholera. In: Diseases of poultry, 13th ed. D. E. Swayne, ed. John Wiley & Sons, Inc. 807. pp. 807–823. 2013.

8. Gower, J. A general coefficient of similarity and some of its properties. Biometrics 27: 857–872. 1971.

9. Leotta, G. A., I. Chinen, G. B. Vigo, J. Gugliada, and M. Rivas. Evaluación de dos técnicas de subtipificación molecular para el estudio de *Pasteurella multocida*. Rev. Argent. Microbiol. 38: 190–196. 2006.

10. R Development Core Team. R: A language and environment for statistical computing. 2008.

11. Sellyei, B., Z. Varga, E. Ivanics, and T. Magyar. Characterisation and comparison of avian *Pasteurella multocida* strains by conventional and ERIC-PCR assays. Acta Vet. Hung. 56: 429–440. 2008.

12. Snipes, K. P., D. C. Hirsh, R. W. Kasten, T. E. Carpenter, D. W. Hird, and R. H. McCapes. Homogeneity of characteristics of *Pasteurella multocida* isolated from turkeys and wildlife in California, 1985-88. Avian Dis. 34: 315–320. 1990.

13. Townsend, K. M., J. D. Boyce, J. Y. Chung, A. J. Frost, and B. Adler. Genetic organization of *Pasteurella multocida cap* Loci and development of a multiplex capsular PCR typing system. J. Clin. Microbiol. 39: 924–929. 2001.

STRATEGIES FOR THE CONTROL OF INFECTIOUS BURSAL DISEASE

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INTRODUCTION

The infectious bursal disease virus (IBDV) infects immature B-lymphocytes in the Bursa of Fabricius (bursa) of chickens and causes an immunosuppressive disease known as infectious bursal disease (IBD) or Gumboro disease. Although some strains of IBDV can cause problems in older birds, IBD is mostly a disease of young chickens. Birds become more resilient to the immune suppression as they get older because the maturing B-lymphocytes that seed secondary lymphoid organs are more resistant to infection. When designing a strategy to control IBD it is important to remember that the immune cells of a young bird are more susceptible to the virus and thus infection at a young age results in a more severe immune suppression.

Secondary infections are often a consequence of the immune suppression caused by IBDV. The immune suppression can also lead to reduced efficacy of vaccination programs for other diseases and clinical reactions from live-attenuated vaccines (1,16). Secondary bacterial infections are a growing concern due to the trend toward reduced or no antibiotic use on poultry farms. In addition to morbidity and mortality, economic losses due to IBDV infections can be manifested in poor feed efficiency, slower growth, uneven weight flocks, longer times to market, and increased processing plant condemnations.

CONTROL OF IBD: STRATEGY AND GOALS

The goal of a good IBD control program should be to prevent immune suppression as well as preventing losses from morbidity and mortality. Since young birds are most susceptible to IBDV infection and immune suppression, establishing adequate maternal immunity in the chicks is a priority. This is accomplished by keeping the antibody titers to IBDV high during the breeder flock laying period. It has been well established that there is a direct correlation between the titer of IBDV antibodies in breeders and the resulting titer of those antibodies in their progeny (13). The quality of maternal antibodies in chicks is equally important as the quantity; they must bind and neutralize the field challenge viruses. Antigenic drift has resulted in a variety of IBDV strains (12) so care must be taken to choose breeder flock vaccines that match the antigenicity of the challenge viruses located on farms where the chicks will be placed.

If the primary goal of producing adequate maternal immunity is met, a secondary goal, to extend that immunity by generating an active immune response in the chicks, should be considered. The half-life of a maternal antibody in a chick is about 3.5 days in broilers (17) and 5.5 days in layers (8), although this varies with type and breed of chicken. The practical result is that most of the maternal antibodies have waned by the time a chicken is 28-35 days of age. At that age, they have become susceptible to IBDV infection and although many of the B-lymphocytes have matured and seeded secondary lymphoid organs, IBDV can still cause a transient immune suppression in these birds (11) that coincides with an active IBDV infection. The immune system will recover most of its normal functions in convalescent birds that have experienced an IBDV infection after three to four weeks of age. In well managed flocks, this transient immune suppression may not be economically significant but in many flocks secondary or opportunistic infections will cause increased morbidity, mortality and processing plant condemnations. Since these secondary infections are often bacterial, the economic hardship can be greater on farms where antibiotics are not used or are in limited use.

Producing an active IBDV immunity in chicks that are experiencing waning maternal antibodies is difficult because the maternal antibodies can interfere with the replication of live-attenuated IBDV vaccines. Timing the administration of a live vaccine is critical to the successful replication of the vaccine virus and the induction of an active immune response. If the vaccine virus is given too soon, it will be neutralized by the maternal antibodies. If given too late, pathogenic field strains of the virus may have already infected and immune suppressed the birds. To complicate this further, the virulence of IBDV vaccine strains is directly related to when they will break through maternal immunity. More virulent (intermediate and intermediate - plus) vaccines will break through higher levels of maternal antibodies to IBDV than the mild vaccines but the tradeoff is increased bursa damage and some degree of immune

suppression. Mild vaccines may be safer but by the time they can be successfully administered, maternal antibodies may be low enough to also allow field virus infections. Monitoring maternal antibody decline in chicks using an ELISA can help with the vaccination timing. Studies using ELISA titers and a mathematical formula called the Deventer Formula have been conducted to determine the best time to vaccinate broilers with IBDV (3,6). This formula has been used successfully with classic vaccine strains of the virus but has not been tested with variant vaccine strains.

Because of the difficulties with producing an active immune response to IBDV in a maternally immune chicken, genetically engineered vaccines for IBDV have been developed (5,18). These vectored vaccines contain the VP2 surface protein gene of IBDV inserted into the genome of the Herpesvirus of turkeys (HVT) and can replicate in the presence of maternal antibodies to IBDV (4). As the HVT-IBD vaccine replicates in the chicken, the VP2 gene is expressed, resulting in the development of an active immunity to IBDV. HVT-IBD vector vaccines can be administered *in ovo* or at hatch, reducing labor costs. Although they have many advantages, these vaccines also have some drawbacks. The active immune response to the IBDV VP2 protein can take time to develop (7). This can be a problem if maternal antibody titers are not high at hatch and leave the chicks susceptible at a young age. Viral interference also appears to be a problem; when vectored vaccines for two different diseases are used together the immune response to one or both can be inadequate.

Immune complex vaccines are another alternative for stimulating active immunity in a flock. These vaccines contain a mixture of vaccine virus and antibody to IBDV and can be given *in ovo* or at the hatchery. The antibodies are bound to the IBDV vaccine virus and greatly reduce viral replication early in the life of the broiler. Thus immune suppression is minimal and often undetectable (9,10). The antibody-antigen complex has been shown to enhance humoral and cellular immunity by targeting the vaccine antigens directly to Fc receptors on antigen processing cells (15). Vaccinating with an immune complex IBD vaccine has been proven to stimulate an active immunity to IBDV even when maternal antibody titers are relatively high (9).

CONTROL OF IBD: WHAT COULD GO WRONG?

Maternal immunity. There are many successful vaccination programs for breeder flocks. It is important to choose one that will keep the maternal antibody titers high throughout the laying period. This will ensure the chicks have a uniformly high titer of maternal antibodies across the population. Uneven titers in the breeder birds will result in a population of chicks that also have varying amounts of antibodies to IBDV. Populating grow out facilities with chicks from multiple breeder flocks can also be the cause of inconsistent antibody titers in the chicks. The subpopulation of chicks that start life with low maternal antibody titers will become infected with IBDV at a young age, become immune suppressed and increase the viral load in the house.

Even when maternal antibody titers are high in the breeder flock, there are still situations that can lead to IBD problems in their progeny. One of the most common is caused by antigenic drift of the field virus (12). Amino acid changes on the surface protein (VP2) could prevent maternal antibodies from binding and neutralizing the virus. These viruses may break through the maternal antibody when the birds are relatively young and cause a permanent immune suppression. To solve this problem, it is necessary to determine the amino acid changes that have occurred in the field virus and then choose a vaccine for use in the breeders that matches the antigenic structure of those field viruses. The molecular procedures used to identify field viruses and determine their VP2 amino acid sequence are fast and inexpensive. When a matching commercial IBD vaccine is not available, producers have had excellent success using killed autogenous vaccines in breeder birds.

Active immunity. The pitfalls of trying to produce an active immunity in birds with maternal antibodies to IBDV have been outlined above. Antigenic drift in field viruses is also a concern when vaccinating to stimulate active immunity. Although there is some cross reaction among the different antigenic strains of IBDV, it is not usually exhibited until the antibody titers in the birds are relatively high. The best vaccine programs will match the antigenic makeup of the vaccine to the pathogenic virus present in the bird's environment. Both the immune complex and HVT-IBD vaccines produce some cross protection among antigenically diverse IBDV strains (7,9,14) but the protection they induce in broilers is expected to be greater against homologous IBDV strains (7).

The window of susceptibility. IBDV is very difficult to eradicate once it is present in the bird's environment (2). Thus, birds are exposed to this endemic virus as soon as they arrive from the hatchery. Even when a control program for IBD is executed flawlessly, there can still be a window when the maternal antibodies are low and antibodies from a vaccine induced active immune response have not yet reached significant titers. This window of susceptibility is an opportunity for field viruses to infect the population. In well managed flocks with low stress and few opportunistic pathogens, the transient

immune suppression that occurs during this window can be manageable.

In conclusion, a successful vaccination program for IBD should be targeted toward the antigenic strains of IBDV that are endemic in the region where the chicks will be placed. These IBDV strains can be identified using molecular sequencing and the data used to select the appropriate vaccines for stimulating maternal immunity. Maternal immunity is finite so protecting the flock with an active immune response to IBDV should also be considered. There are several types of IBD vaccines available for stimulating an active immune response and choosing the best one should be done on a case-by-case basis.

REFERENCES

1. Becht, H. Infectious bursal disease virus. Curr. Top. Microbiol. Immunol. 90: 107–121. 1981.

2. Benton, W. J., M. S. Cover, J. K. Rosenberger, and R. S. Lake. Physiochemical properties of the infectious bursal agent. Avian Dis. 11: 438–445. 1967.

3. Block, H., K. Meyer-Block, D. E. Rebeski, H. Scharr, K. R. S. de Wit, and S. Rautenschlein. A field study on the significance of vaccinations against infectious bursal disease virus (IBDV) at the optimal time point in broiler flocks with maternally derived IBDV. Avian Pathol. 36: 401–409. 2007.

4. Bublot, M., N. Pritchard, F.-X. LeGros, and S. Goutebroze. Use of a vectored vaccine against infectious bursal disease of chickens in the face of high-titired maternally derived antibody. J Comp Path 137: S81–S84. 2007.

5. Darteil, R., M. Bublot, E. Laplace, J. F. Bouqet, J. C. Audonnet, and M. Riviere. Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. Virology 211: 481–490. 1995.

6. De Herdt, P., E. Jagt, G. Paul, S. Van Colen, R. Renard, C. Destrooper, and G. van den Bosch. Evaluation of the enzyme-linked immunosorbent assay for the detection of antibodies against infectious bursal disease virus (IBDV) and the estimation of the optimal age for IBDV vaccination in broilers. Avian Pathol. 34: 501–504. 2005.

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

8. Gharaibeh, S., and K. Mahmoud. Decay of maternal antibodies in broiler chickens. Poult. Sci. 92: 2333–2336. 2013.

9. Haddad, E. E., Whitfill, C. E., Avakian, A. P., C. A. Ricks, P. D. Andrews, J. A. Thoma, and P. S. Wakenell. Efficacy of a novel infectious bursal disease virus immune complex vaccine in broiler chickens. Avian Dis. 41: 882–889. 1997.

10. Hassanzadeh, M., Fard, M. H. B., and Tooluo, A. Evaluation of the immunogenisity of immune complex infectious bursal disease vaccine delivered in ovo to embryonated eggs or subcutaneously to day-old chicks. Int. J. Poult. Sci. 5: 70–74. 2006.

11. Hoerr, F. J. Clinical aspects of immunosuppression in poultry. Avian Dis. 54: 2–15. 2010.

12. Jackwood, D. J., and S. E. Sommer-Wagner. Amino acids contributing to antigenic drift in the infectious bursal disease Birnavirus (IBDV). Virology 409: 33–37. 2011.

13. Loeken, M. R., and T. F. Roth. Analysis of maternal IgG subpopulations which are transported into the chicken oocyte. Immunology 49: 21–28. 1983.

14. Perozo, F., P. Villegas, J. Cruz, and N. Pritchard. Efficacy of single dose recombinant herpesvirus of turkey infectious bursal disease virus (IBDV) vaccination against a variant IBDV strain. Avian Dis. 53: 624–628. 2009.

15. Pham, G. H., B. V. Iglesias, and E. J. Gosselin. Fc receptor-targeting of immunogen as a strategy for enhanced antigen loading, vaccination, and protection using intranasally administered antigen-pulsed dendritic cells. Vaccine 32: 5212–5220. 2014.

16. Saif, Y. M. Immunosuppression induced by infectious bursal disease virus. Vet. Immunol. Immunopathol. 30: 45–50. 1991.

17. Skeeles, J. K., P. D. Lukert, O. J. Fletcher, and J. D. Leonard. Immunization studies with a cell-culture adapted infectious bursal disease virus. Avian Dis. 23: 456–465. 1979.

18. Tsukamoto, K., S. Saito, S. Saeki, T. Sato, N. Tanimura, T. Isobe, M. Mase, T. Imada, N. Yuasa, and S. Yamaguchi. Complete, long-lasting protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. J. Virol. 76: 5637–5645. 2002.

DETERMINATION OF LETHAL EFFECTS OF *ENTEROCOCCUS FAECALIS* INFECTION IN DEVELOPING CHICKEN EMBRYOS

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ABSTRACT

Enterococcus faecalis is an inhabitant of chicken gut microflora which act as an opportunistic pathogen. Our previous studies demonstrated that *Enterococcus* is the predominant bacteria isolated from dead embryos in commercial hatcheries in western Canada. Very little is known regarding the pathogenesis of *E. faecalis* infection in poultry.

The objective of this study was to investigate the ability of *E. faecalis* to cause embryonic lethality. We hypothesized that *E. faecalis* can enter the egg through a temperature gradient by simulating a real-life scenario wherein warmer eggs come into contact with cooler and bacteria is sucked into eggs. Incubating broiler eggs (at day 12 of incubation) were randomly allocated into treatment groups (n=40/treatment). Eggs were initially at 37˚C and later dipped for 30 seconds in a 10^9 CFU/mL bacterial suspension and maintained at 10˚C. Three *E. faecalis* strains were tested; isolated from the rectum of a healthy 32 week old broiler breeder, a multidrug resistant strain isolated from an early dead embryo at a commercial broiler hatchery and one strain isolated from the yolk sac of a three day old chick with yolk sac infection. Embryo viability was determined at 48 hours post infection, six days post infection and at hatch. Live embryos (n=5) were euthanized and bacteriological examination and histopathology were performed on intestine, yolk, liver and brain as well as from any dead embryos from each group and each time point.

Cumulative embryonic mortality at the time of hatch were 16.67%, 40.48%, 40.48% and hatchability was 47%, 50% and 46.15 % respectively for the three strains mentioned. The *E. faecalis* re-isolation rate was highest in the multidrug resistant strain infected embryos where 71.43% of embryos were positive 48 hours after infection. The *E. faecalis* isolation percentages were 57.14%, 71.43%, 28.57%, 42.86% for intestine, yolk, liver and brain respectively.

This pattern was consistent at six days post infection where the highest bacterial isolation was observed in the same group where at least one organ was positive for bacterial isolation. Isolation rate increased with the day of incubation in all the treatment groups. Histopathology revealed colonized gram positive cocci in the yolk in high numbers in dead embryos compared to live embryos.

These findings suggest that *E. faecalis* can invade and colonize in developing chicken embryos and can cause lethal effects on them.

INTRODUCTION

Enterococci are normal gastro intestinal inhabitants of human and animals. They are gram positive, non-spore forming facultative anaerobes and a major cause of nosocomial infections which leads to bacteremia, endocarditis, and urinary tract infections in human (8). Growing multi drug resistance including vancomycin resistance, more particular in *E. faecalis* and *E. faecium* causes therapeutic failures (2). *Enterococcus* species inhabit in the chicken gut just after they hatch. *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. casseliflavus*, and *E. gallinarum* are the predominant inhabitants. Enterococci become opportunistic pathogens when they encounter enteric infections or any condition compromising the intestinal villous epithelium, allowing penetration of resident enterococci to systemic circulation and can result in septicemia, bacterial endocarditis, or both. *E. faecalis* is associated with amyloid arthropathy and *E. cecorum* causing osteomyelitis can lead to significant economic loses in the poultry industry (5, 7). *E. hirae* and *E. durans* have been associated with brain necrosis and encephalomalacia in young chicks (1). Egg transmission or fecal contamination of *Enterococcus* spp. in hatching eggs resulted in embryo mortality and an increased number of chicks unable to penetrate through the shell at hatch. Omphalitis or enlarged yolk sacs may be seen in chicks infected at hatching (4).

Recently, the incidence of *Enterococcus*-associated yolk sac infections has been emerging in the poultry industry worldwide, including Canada. We hypothesized that the increased incidence of *Enterococcus*-associated embryo mortality is predisposing to the increased incidence of *Enterococcus*-associated yolk sac infections. Our previous work supported this observation where we have found that the majority of chicken embryonic

death in commercial hatcheries in western Canada is associated with *Enterococcus* species (6).

In the past, *E. coli* accounted for the majority of embryonic deaths compared to *Enterococcus* species (3). The emergence of *Enterococcus* species associated yolk sac infections and embryonic deaths may be attributed to their mutations in virulence genes which were non-pathogenic in chickens in the past.

Despite the acknowledged status of *Enterococcus* species as a poultry pathogen, the exact mode of transmission, port of entry, and virulence mechanisms in broiler chicken embryo mortality are largely unknown. Therefore we have conducted an infection model to determine the pathogenesis of *Enterococcus*-associated chicken embryonic mortality.

MATERIAL AND METHODS

Specific pathogen free eggs were incubated and challenged at day 12 of incubation in this experiment. All of the eggs were checked for embryo viability before challenge. The principle of the infection model was to facilitate bacterial penetration through the egg shell when a temperature gradient developed between the eggs and bacterial broths. The viable eggs were divided into three groups (n=40) and were dipped in three *E. faecalis* bacterial broths individually. The three *E. faecalis* strains tested were; isolated from rectum of a healthy 32 week old chicken which was regarded as a negative control strain, multidrug resistant strain isolated from an early dead embryo at a commercial broiler hatchery and one strain isolated from the yolk sac of 3 day old chick with yolk sac infection. Three liters of 10^9 CFU/mL bacterial broths were prepared for each strain mentioned above and cooled down in to 10°C. The egg temperature was assumed to be 37°C which is the incubator temperature. The eggs from each group were immersed in the respective bacterial broth for 30 seconds thereafter the eggs were held for a few second to air dry. The eggs were then placed back into the incubators until hatch. Embryo and chick viability were determined at 48 hours after challenge, six days after challenge and at the day of hatch. Bacteriological swabs and samples for histopathology were taken from intestines, liver, yolk and brain at 48 hours, six days post infection and at hatch from five viable embryos and five dead embryos from each time point from each treatment groups. Cumulative embryo mortality and hatchability was determined at the day of hatch. Bacteriological swabs were inoculated on 5% Columbia sheep blood agar and m-Enterococcus selective agar, incubated at 37°C for 24 to 48 hours. The isolated colonies were identified using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

RESULTS

The cumulative embryo morality percentages were 16.67%, 40.48%, 40.48% for the control strain isolated from the rectum of a healthy chicken, multidrug resistant strain and yolk-sac infection associated (field) strain respectively. The number of embryos which died at 48 hours, six days post infection and at hatch for control strain was 0, 0, 7, and for multidrug resistant strain 2, 0, 15 and for the field strain 2, 1, and14 respectively for each time point. The hatchability was 47%, 50% and 46.15 % respectively for the three strains mentioned. MALDI-TOF MS identification confirmed the presence of *E. faecalis* from cultured samples and the absence of any contaminants. The *E. faecalis* re-isolation rate was highest in the multidrug resistant strain infected embryos where 71.43% of embryos were positive at 48 hours after infection. The *E. faecalis* isolation percentages were 57.14%, 71.43%, 28.57%, 42.86% for intestine, yolk, liver and brain respectively.

This pattern was consistent at six days post infection where the highest bacterial isolation was observed in the multidrug resistant strain infected group where 100% were infected, while the control strain and field strain were 60% and 83.33% respectively. In the multidrug resistant strain infected group, at least one organ was positive for bacterial isolation. Bacterial load in examined organs increased with the day of incubation in all the treatment groups. There were no significant gross pathological changes in dead embryos during the incubation period. Histopathology revealed colonized gram positive cocci in yolk, brain and liver in high numbers in dead embryos compared to live embryos.

DISCUSSION

We were able to develop an infection model to reproduce *E. faecalis* associated chicken embryo mortality. *E. faecalis* was able to penetrate and colonize in developing chicken embryos leading to reduced hatchability and was able to cause mortality of chicken embryos. The highest colonization in visceral organs by the multidrug resistant strain may associated with virulence factors which harbors in transmissible plasmid or pathogenicity island which encodes antimicrobial resistance. We have observed that the number of embryonic deaths increased with day of incubation and this observation is consistent with increasing bacterial load in visceral organs. Since we didn't observe any gross pathological lesions in the dead embryos, the mechanisms of embryo death is

uncertain. We hypothesized that severe bacterial load interferes with the normal embryogenesis pathway and leads to death. We observed the majority of dead embryos had retained yolk sac. This may be explained by the high bacterial load in the yolk interfering with yolk metabolism and nutrition uptake by the embryo leading to embryonic death.

Even though we have experimentally proved that *E. faecalis* was able to cause chicken embryo mortality, the virulence factors and pathogenic mechanisms of embryonic death are not yet understood. We are in the process of determining the most prevalence virulence factors and virulence gene expression levels of *E. faecalis* in developing chicken embryos. Simultaneously, cytokine expression levels in the chicken embryo will be analyzed to determine host reaction to bacterial invasion.

REFERENCES

1. Abe, Y., K. Nakamura, M. Yamada, and Y. Yamamoto. Encephalomalacia with Enterococcus durans infection in the brain stem and cerebral hemisphere in chicks in Japan. Avian diseases 50:139-141. 2006.

2. Arias, C. A., and B. E. Murray. The rise of the Enterococcus: beyond vancomycin resistance. Nature reviews. Microbiology 10:266-278. 2012.

3. Babaca, Z. Epidemiological and Bacteriological Studies on Dead-in-Shell Embryos. J Veterinar Sci Technol 5:2. 2014.

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

5. Jung, A., and S. Rautenschlein. Comprehensive report of an Enterococcus cecorum infection in a broiler flock in Northern Germany. BMC veterinary research 10:311. 2014.

6. Karunarathna, R., S. Popowich, M. Wawryk, B. Chow-Lockerbie, K. A. Ahmed, C. Yu, M. Liu, K. Goonewardene, T. Gunawardana, and S. Kurukulasuriya. Increased Incidence of Enterococcal Infection in Nonviable Broiler Chicken Embryos in Western Canadian Hatcheries as Detected by Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry. Avian Diseases 61:472-480. 2017.

7. Landman, W. J. M. Amyloid arthropathy in chickens: (Summary of thesis, Utrecht University, faculty of veterinary medicine, 1998). Veterinary quarterly 21:78-82. 1999.

8. Moellering Jr, R. C. Emergence of Enterococcus as a significant pathogen. Clinical infectious diseases:1173-1176. 1992.

BARCODE-LABELLING SYSTEM FOR SURGE CAPACITY LANNING EXERCISE ON HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) VIRUS

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SUMMARY

Laboratory diagnosis of avian influenza has become a cornerstone for the prevention, containment and surveillance strategies of this disease. During a suspected or actual HPAI outbreak, the key goals of a response are: meeting the surge requirements for diagnostic testing, and to report all diagnostic test results to appropriate personnel and information management systems as soon as possible. In the present studies, we evaluated the testing capacity of our laboratory through simulation and modeling of the methodologies and integration of barcode-labelling system in the USDA-approved real-time PCR detection platforms to handle 400 randomly spiked avian influenza virus specimens.

Highly pathogenic avian influenza (HPAI) viruses caused unprecedented outbreaks in several commercial poultry, backyard flocks, captive wild birds, and wild birds in 21 states during 2014-2016. These outbreaks killed approximately 43 million chickens and 8 million turkeys either due to infection or depopulation as part of eradication effort (1). Early detection of avian influenza is very critical to initiate efficient control and eradication programs. Georgia is one the leading poultry-producing states in the US. Occurrence of HPAI would cause very devastating effects on the economy of this state. During an emergency, there is a sudden and sustained increase in the volume of testing that demands substantial operational changes in the clinical laboratories. Evaluation of laboratory surge capacity by "simulation exercise" is a valuable way of putting into practice response plan prior to an actual need. These types of exercises create confidence among staff in a low stress environment, provides an opportunity to explore their roles and expectations. Further, these exercises help identify functional inconsistencies that can be addressed by providing specific training and process improvement. Methods:

Criteria for zone testing. Under OIE Code definitions, an "infected are" means a clearly defined territory in which a disease has been diagnosed. This "Hot zone" is two-mile radius around the index case. In this zone there is intense testing and mass depopulation measures are decreed by veterinary authorities. Another zone, called "surveillance zone", is drawn around the radius of six miles from the index case. In this zone, all the farms are tested every seven days, and the movements of birds are strictly under permit. The zone outside this six-mile radius is regarded as "AIV -free zone."

During an AI event, the number of samples submitted from any zone under consideration is directly related to the population density of susceptible birds in that area. It has been estimated that in NE Georgia, there are 410 poultry houses with 8.44 million birds in a radius of six miles. In addition, there are "panic submissions" mostly originating from AIV-free zones. In this situation it is extremely important to correctly identify specimens to avoid costly human errors.

Operations in the Receiving Area of laboratory. The aim is to correctly assign a specimen label in 12x8 rack to define the locus of the specimen for all the down-stream procedures, like RNA extraction, PCR set-up and for the data entry in Laboratory Information Management System (LIMS).

Receiving personnel are responsible for assigning the priority for each sample in a prescribed form, data entry into LIMS database and printing duplicate barcode labels for each specimen tube. All the procedures from this point onward shall be performed in a designated class II bio-safety cabinet (BSC).

Operations in the BSC. This step involves opening the packages, surface decontamination of specimen tubes, arranging the specimens according to the assigned priority. Barcode labels are affixed to individual specimens of brain heart infusion broth (BHI) tubes are affixed barcode labels and arranged in 12x8 format in designated racks. Another barcode label is affixed on a designate Form at the same time. Once the rack is full, the barcode from each tube is scanned to enter the accession number in a formatted spreadsheet that shows the position of each specimen in a horizontal as well as vertical format (Fig. 1, 2). This spreadsheet is uploaded on the network drive to facilitate direct downloading of accession numbers into the specific instrument software in the BSL3 lab. This step avoids specimen labelling errors.

Operations within the BSL3 laboratory:

1. To issue the chain of custody of specimens by scanning the barcode Form prepared in the Receiving.

2. Sample rack is sent directly to BSL3 area through the pass-through chamber.

3. The arrangement of BHI tubes in 12x8 format is followed through-out the RNA extraction and PCR-set-up procedures (Fig. 3).

4. Once the PCR run is over, barcode label Form are used again to enter the results in the LIMS (Fig. 5).

MATERIAL AND METHODS

Four hundred and twenty-five BHI samples were randomly spiked with inactivated H6N2 virus at three different pre-determined dilutions. All the procedures were performed according to NVSL SOP-AV-068.

RESULTS

Five trained technicians completed the task of testing and closing all the accession in 10 hours. Standard operative procedures for each set of operation was streamlined to reflect the actual task performed. All the randomly-spiked specimens were correctly identified from each plate, with exception of plate that showed cross-contamination in one well (Fig. 3, 4, and 5).

CONCLUSION

Prioritizing "Rush" cases is very important for monitoring new cases as well as for movement of birds under permit. Barcode labelling and arranging the specimens in 12x8 format ensures the uniformity of sample labelling throughout the RT-PCR procedures. Preparation of barcode label form facilitated chain of custody, accession closing and avoided costly human errors of data entry.

REFERENCES

1. Final Report for the 2014-2015 Outbreak of Highly Pathogenic Avian Influenza (HPAI) in the United States, Revised August 11, 2016, Veterinary Services, United States Department of Agriculture.

Figure 1. Barcode scanning of specimen tubes and preparation of barcode-label Forms.

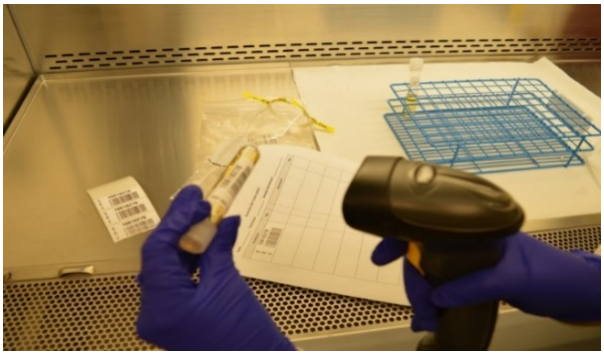


Figure 2. Barcode-scanned tubes are arranged in 12x8 spreadsheet.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	**10788946**	**10788947**	**10788948**	10788949	10788950	10788951	10788952	10788953	10788954	10788955	10788956	10788957		
2	10788958	10788959	10788960	10788961	10788962	10788963	10788964	10788965	10788966	10788967	10788968	10788969		
3	10788970	10788971	10788972	10788973	10788974	10788975	10788976	10788977	10789149	10789152	10789154	10789156		
4	10789158	10789159	10789536	10789537	10789538	10789539	10789540	10789541	10789542	10789543	10789544	10789545		
5	10789546	10789547	10789548	0	0	0	0	0	0	0	0	0		
6	0	0	0	0	0	0	0	0	0	0	0	0		
7	0	0	0	0	0	0	0	0	0	0	0	0		
8	NEC	NTC								PAC	PAC	PEC		
9														
10													1	10788946
11													2	10788947
12													3	10788948

Figure 3. Tube order of specimens is maintained in all the down-stream procedure.

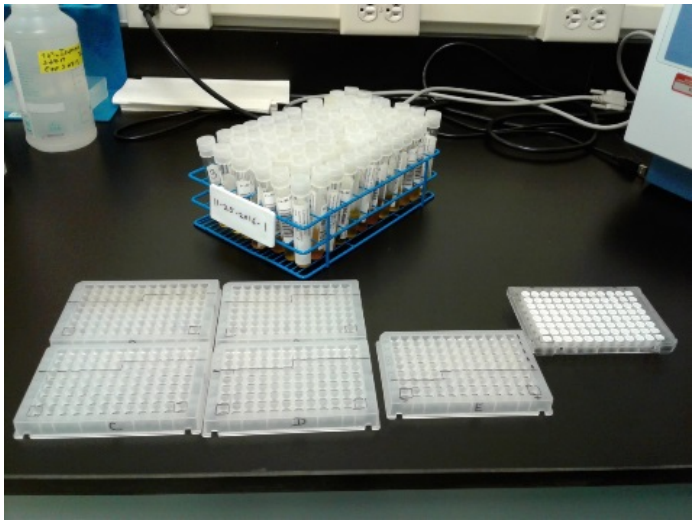
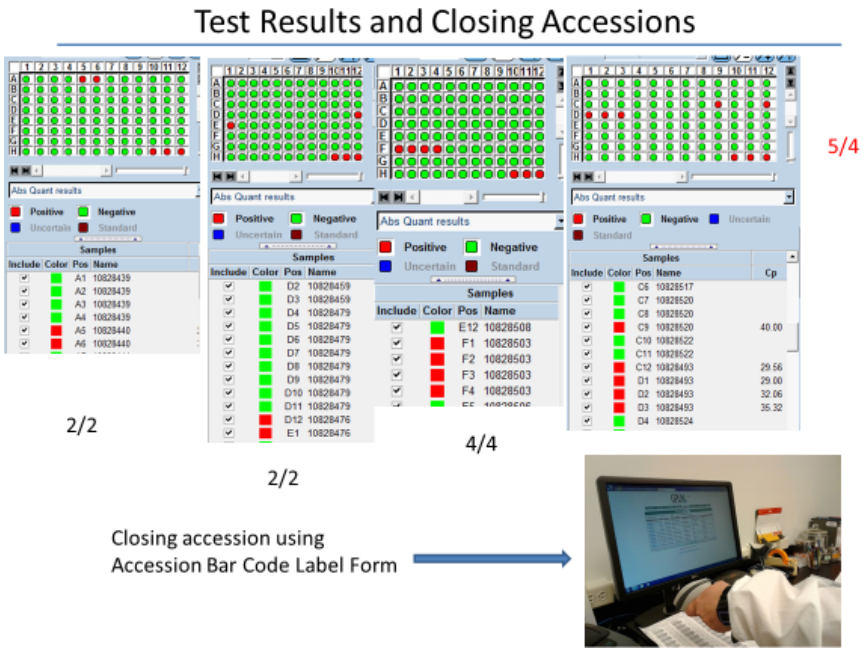


Figure 4. Trial with 425 randomly-spiked samples.

425 Sample exercise spiked with A/chicken/CA/1255/2010/H6N2

Plate	Accession	# tubes	Concentration	Ct value
1	10828440	2	Strong, 10^{-3}	28.61
			Moderate, 10^{-4}	31.71
2	10828476	2	Moderate, 10^{-4}	31.76
			Weak, 10^{-5}	35.1
3	10828493	4	Strong, 10^{-3}	29.56
			Moderate, 10^{-4}	29
			Weak, 10^{-5}	32.06
			Strong, 10^{-3}	35.32
4	10828503	4	Strong, 10^{-3}	28.11
			Moderate, 10^{-4}	31.91
			Weak, 10^{-5}	35.09
			Strong, 10^{-3}	28.25

Figure 5. Results of twelve AIV-positive samples.



USE AND INTERPRETATION OF ACTIVE SEROLOGICAL SURVEILLANCE IN PROCESSING AGE BROILERS VIA INFECTIOUS BRONCHITIS AND NEWCASTLE DISEASE ELISA

J. A. Linares and M. Putnam

Serology can be used for passive and active surveillance. Passive surveillance is used when flocks are sick or not performing as expected. This type serology is passive in the sense that we wait to test until there is a problem. Comparing results between acute and convalescent serum could help in the diagnostic process. On the other hand, active surveillance consists of scheduled monitoring of flocks at key times such as evaluation of processing age titers. Active surveillance is carried out even when the flocks appear to be healthy. Processing age antibody titers give us a window into the response to vaccination programs and/or field challenges.

As part of our technical support for respiratory vaccines we initiated a program of active serological surveillance of processing age broilers via infectious bronchitis (IB) and Newcastle disease (ND) ELISA. The monitoring program consists of testing 11 serum samples per flock, at least two flocks per week per complex at the customer's diagnostic laboratory of choice. The results help us to establish titer patterns for a specific production complex. Results are evaluated primarily by comparing geometric mean titer (GMT) and coefficient of variation (CV) data from all the flocks tested.

Following are results to date from two customers participating in our active serological surveillance program. Both customers opted to have their serology conducted at the same laboratory via IDEXX ELISA.

RESULTS

Customer A. IB and ND are administered at the hatchery via aqueous spray. This customer is using our GA08 vaccine as the only IB vaccine in an area with documented GA08 and GA13 challenge. During this testing period they used two mild Newcastle vaccines alternatively. Their GMTs for IB and ND are consistently low (Figure 1 and Figure 2). The results are compatible with IB and ND titers close to processing age (~40 days-old) associated with a hatchery-only vaccination program and field challenge under control. The results are also compatible with bird performance. The first flocks on our GA08 vaccine were processed around week 33 of 2016 (circled on Figure 3). The weekly % condemns improved since then and remained low into 2017.

Customer B. This complex had been on a Mass, Del072, Ark and ND B1 at the hatchery followed by field boosting with the same vaccine combination. While on this program the flocks were experiencing respiratory issues associated with GA13 challenge and rolling Ark reactions. The customer changed the vaccination program to our GA08, Mass and a milder ND. The vaccines were administered at the hatchery via aqueous spray with no field boosting. The customer shared processing age serology results from flocks on their previous vaccination program and after the switch to GA08 and Mass. The first flock tested on the GA08 and Mass program is circled on Figure 4. After the switch to GA08 and Mass, the IB GMTs changed to a lower pattern and bird performance improved. The ND GMTs remained consistently low (Figure 5).

CONCLUSION

Active serological surveillance for IB and ND is a useful tool to help evaluate the antibody response to vaccines and /or field challenges. The results should be interpreted in the context of the vaccination program, known respiratory challenges, overall flock health and flock performance.

Figure 1. Customer A, IB serology, Oct-Dec 2017; ~ 40 day-old broilers.

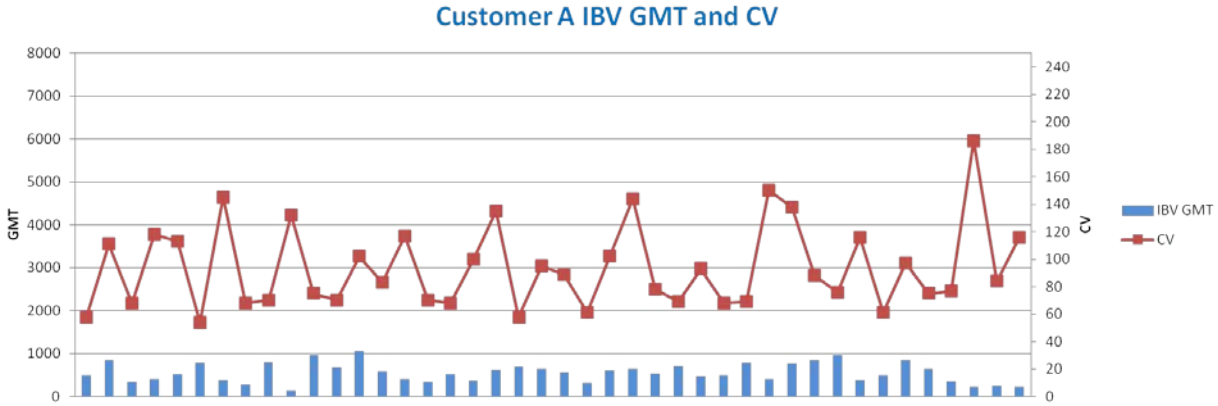


Figure 2. Customer A, ND serology, Oct-Dec 2017; ~ 40 day-old broilers.

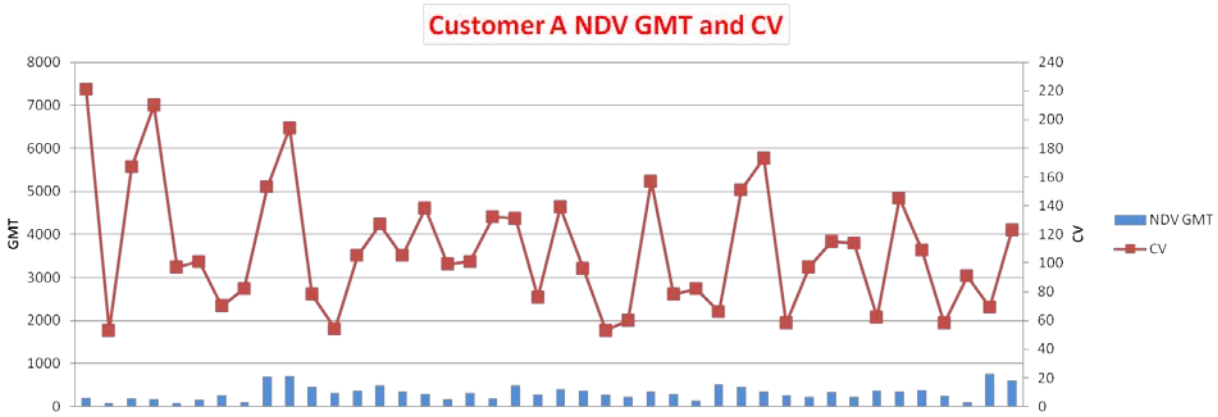


Figure 3. Customer A, three years of weekly % condemns data.

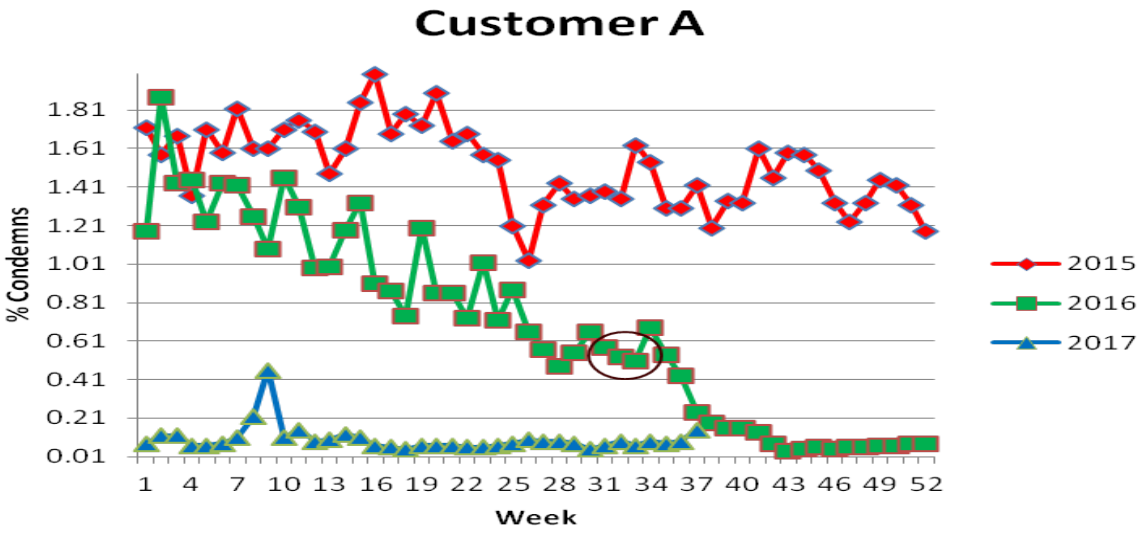


Figure 4. Customer B, IB serology, Jun-Nov 2017; ~ 60 day-old broilers.

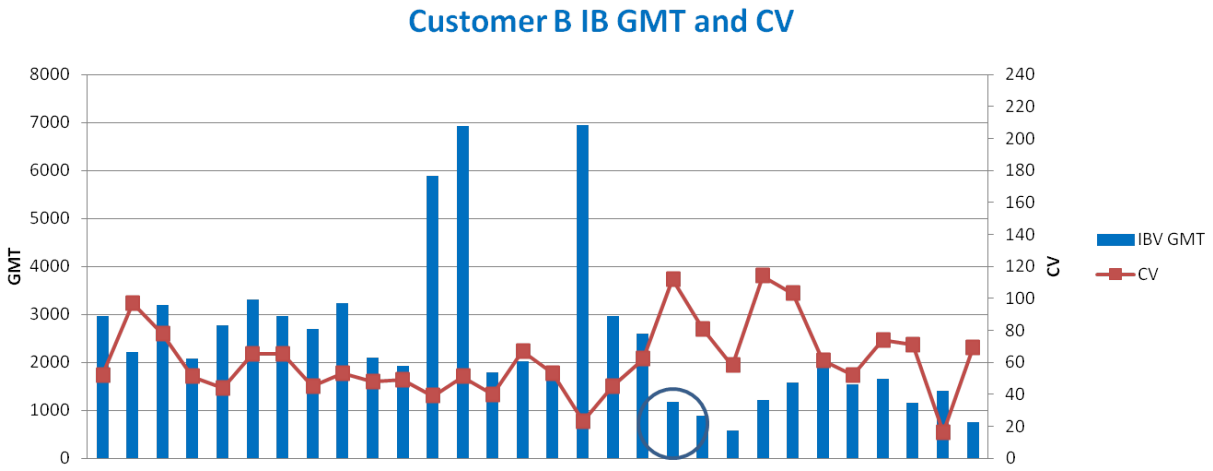
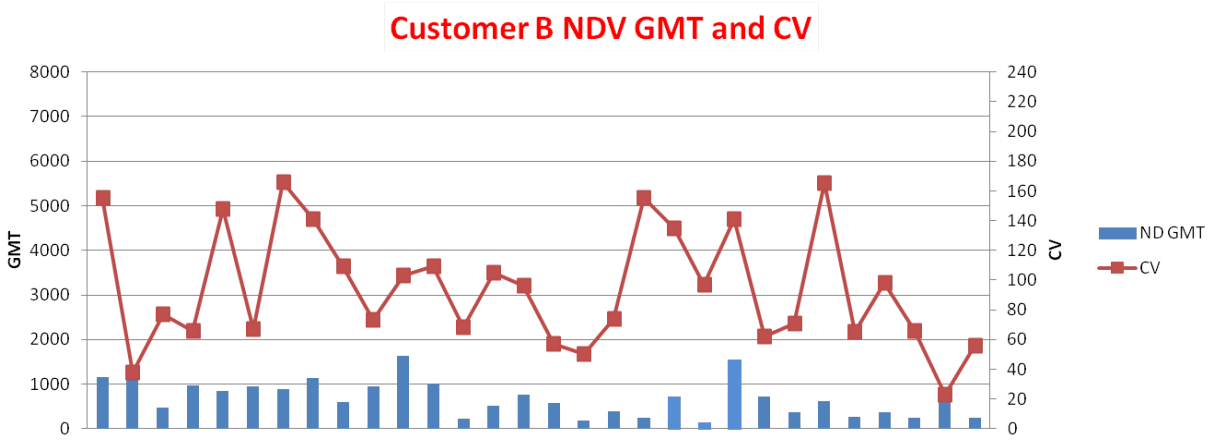


Figure 5. Customer B, ND serology, Jun-Nov 2017; ~ 60 day-old broilers.



REOVIRAL TENOSYNOVITIS OUTBREAK IN THE FRASER VALLEY, BRITISH COLUMBIA

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SUMMARY

Since the beginning of 2016, there has been a substantial increase in the number of reoviral tenosynovitis cases in the Fraser Valley, British Columbia. By the end of 2017, over one hundred cases of reoviral tenosynovitis were diagnosed in the region (Fig. 1). The reovirus outbreak in the Fraser Valley has caused increased mortality, viral arthritis/tenosynovitis, and poor performance (including poor feed conversions, lack of uniformity and reduced weight gain), resulting in significant economic losses to the broiler industry

Avian reovirus, first described in the 1950s, is a double-stranded RNA virus that is ubiquitous in commercial poultry (3). The majority of reovirus strains is non-pathogenic and appears to survive harmlessly in the intestine while some strains are associated with various diseases such as viral arthritis/tenosynovitis, myocarditis, enteric diseases, immunosuppression, and malabsorption syndromes (1, 3). The reovirus outbreak in the Fraser Valley is caused by a variant of reovirus that affects intestines, hearts, joints, and tendons. The virus was first isolated in the region from chicks that originated outside of the province. Reovirus can be transmitted both vertically and horizontally (2, 4). The primary source of contact infection is fecal contamination (1). Fomites can be an important source of contamination since the virus is quite resistant to inactivation and can persist on farm materials for many days or weeks (2).

Clinical signs were reported in broiler chickens between 9-37 days old. The majority of the cases were reported from three to four weeks of age as the birds begin to experience substantial weight gain and display lameness. Common reported clinical signs included swollen hock joints, unilateral or bilateral splayed legs, lack of uniformity, and delayed weight gain.

Diagnosis of reoviral tenosynovitis is confirmed with gross pathology, histology, and PCR findings. Postmortem examination showed edematous and ruptured digital flexor tendons. Excessive serosanguinous fluid in the hock joints is often observed. It is not uncommon to find affected chickens with bruises on the wings and sternal bursitis since the birds struggled to walk. Pale, toneless small intestines were observed in some cases. Microscopic lesions in the digital flexor tendon were characterized by diffuse thickening of the synovium with synoviocyte hypertrophy and inflammation of the synovium composed of a high number of lymphocytes, plasma cells and macrophages with fewer heterophils. Formation of lymphoid nodules within the connective tissue was often reported. In some cases, opportunistic bacteria such as *Staphylococcus* spp. and *E. coli* were isolated from the joints.

There is no effective treatment for reovirus infection. Affected birds showing severe signs of lameness should be euthanized. Secondary bacterial arthritis may be managed with antibiotics based on culture and sensitivity results. The broiler breeders in the Fraser Valley are vaccinated with a commercial reovirus vaccine. However, the vaccine does not provide cross protection to this new reovirus variant. The development of a new autogenous vaccine is underway for this new reovirus isolate. Biosecurity, proper cleaning and disinfection continues to remain the most important means of control and prevention of reoviral tenosynovitis. Farms with recurrent reovirus infection were often associated with on-farm manure storage, poor ventilation, and sub-optimal brooding conditions.

REFERENCES

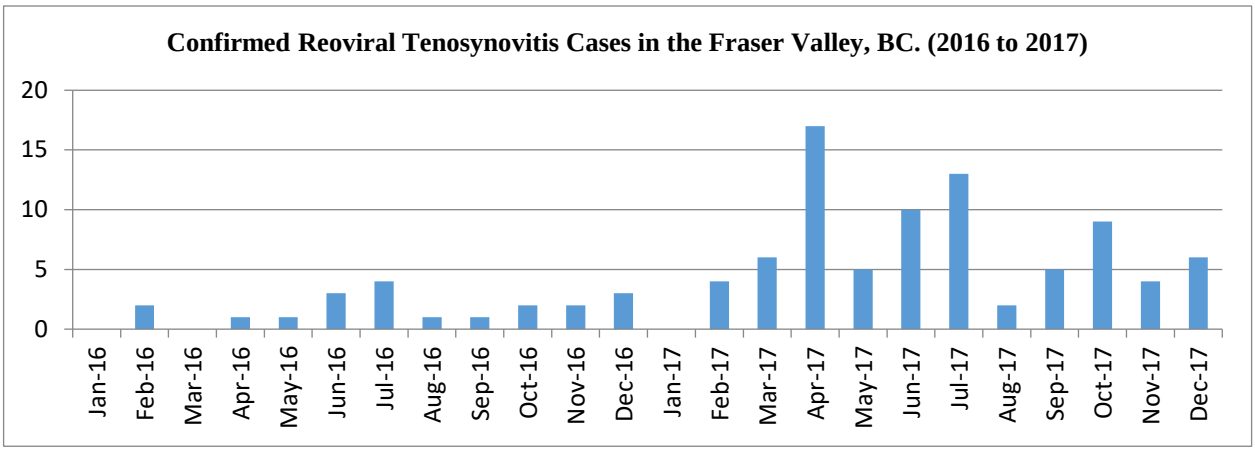
1. Rosenberger, J. K. Reovirus Infection. In: Diseases of Poultry, 11th ed. B. W. Clark, ed. Iowa State University Press, Ames, IA. Pp. 283-290. 2003.

2. Sahu, S. P., Olson, N. O. Comparison of the characteristics of avian reoviruses isolated from the digestive and respiratory tract with viruses isolated from the synoviae. Journal of Vet. Res. 3: 847-850. 1996.

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

4. Van der Heide, L., Kalbac, M. Infectious Tenosynovitis (Viral Arthritis): Characterization of a Connecticut Viral Isolant as a Reovirus and Evidence of Viral Egg Transmission by Reovirus-Infected Broiler Breeders. Avian Dis. 19: 683-688. 1975.

Figure 1. Number of confirmed reoviral tenosynovitis cases in 2016-2017 from a poultry practice in Fraser Valley, BC.



A CASE REPORT: NECROTIC ENTERITIS OUTBREAK FOLLOWING SMOKE INHALATION IN A COMMERCIAL BROILER FLOCK IN ABBOTSFORD, BRITISH COLUMBIA

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SUMMARY

At 5:30 p.m. on August 9th, 2017, a massive industrial fire broke out at a lumber mill approximately 700 meters away from a commercial broiler farm in Abbotsford, British Columbia (Figure 1). The fire lasted over 10 hours, and generated a thick plume of smoke in the surrounding area. The next morning, a sudden increase in mortality was reported from the broiler farm. Ten dead nine day old RWA (raised without antibiotics) broiler chickens were submitted for diagnostic work up. The flock size of 5,500, housed in one of the four floors, also exhibited a decline in water and feed consumption overnight. Smoke was observed inside the barn from on-farm cameras.

Postmortem examination revealed friable small intestine distended with watery contents, gas, and mucoid exudates. The intestinal mucosa was roughened and covered with a pseudomembrane. The gizzard was packed with sawdust with a small amount of feed. Sloughing of the koilin membrane was also noted. Histologically, multifocal heterophilic infiltrate, acute hemorrhage, and dense aggregates of yeast were observed. Necrotic enteritis was diagnosed.

Treatments with water acidification, hydrogen peroxide, and amprolium were administered. Over 300 mortalities (5.45%) were reported between August 10th and August 23rd. The increase in mortality eventually subsided 13 days after the initial incident. The three other floors in the same barn, which consists of one floor of RWA broilers and two floors of regular broilers were mildly affected by the incident.

In addition to being raised without antibiotics, the affected flock was different from the rest of the barn as it was previously isolated with *Salmonella* Enteritidis, which could have had played a role in complicating the impact of necrotic enteritis. This case had demonstrated a necrotic enteritis outbreak triggered by a stressful event (smoke inhalation) that had led to subsequent feed disruption, immunosuppression, and impaired gut health.

The presentation will focus on risk factors that contribute to the development of necrotic enteritis. Prevention strategies, especially those that are applicable to chickens raised without antibiotics, will also be discussed.

Figure 1. Image retrieved online from CTV News Vancouver on August 10, 2017.

A fire at a lumber business in Abbotsford is seen in this image from Wednesday, Aug. 9, 2017.

A CASE REPORT: *TRICHOMONAS GALLINAE* OUTBREAK IN A PIGEON FARM IN MISSION, BRITISH COLUMBIA

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SUMMARY

In early September, 2017, four adult pigeons (two live and two dead) and three dead squabs were submitted for a diagnostic workup from a squab farm in Mission, British Columbia. The farm, with a flock size of 5,000 adult pigeons and 1,000 squabs, had experienced an increase in both adult and squab mortality for over three months. Five to ten adult pigeons were found dead per day. Squab mortality was as high as 90%. The majority of the squabs found dead were between one to three days old. The two live adult pigeons submitted were depressed and underweight. Light brown, raised lesions were observed in the oral cavity. At necropsy, all birds had a diffusely thickened, white crop and esophageal mucosa. Histological findings of the crop and esophagus revealed thickening of the epithelium due to epithelial hyperplasia, and infiltration with lymphocytes and macrophages. Protozoal trophozoites were identified from the liver, crop, and esophagus. PCR was positive for *Trichomonas gallinae*. *Salmonella* serogroup B was also cultured.

The flock was treated with copper sulfate (0.4mg/mL) in the drinking water for five days. Approximately eighty severely sick pigeons were isolated and were treated with oral antibiotic twice daily for five days. The number of pigeon mortality was reduced to 50% after a course of antibiotic treatment; however, the number of squab mortality failed to subside. Final *Salmonella* serotyping confirmed that *Salmonella* Typhimurium was isolated.

Salmonella infection has likely contributed to the treatment challenge in this case. Due to the limited use of antibiotics in meat pigeons, management practice becomes the most important aspect in this case. Water sanitation, water acidification, biosecurity, cleaning and disinfection of the premise were recommended. To date, this is still an ongoing case and the clinical outcome is yet to be determined.

VARIANT INFECTIOUS BURSAL DISEASE VIRUS (VARIBDV)-SK09: A POTENTIAL VACCINE CANDIDATE TO CONTROL IBDV INFECTION IN CANADA

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SUMMARY

Infectious bursal disease virus (IBDV) circulating in North America are mainly variant strains (varIBDV) that induce severe immunosuppression. varIBDV strains are not amenable to commercially available vaccines. Previously, we have demonstrated the potential of a locally prevalent strain, varIBDV-SK09, to be vaccine candidate. The objective of the present study was to develop a breeder vaccine based on varIBDV-SK09 to control varIBDV infection in Canada. Four groups of breeders (n=20 per group) were vaccinated with inactivated, live, live prime plus inactivated boosters, or immune complex varIBDV-SK09, respectively. An additional group received a commercially available vaccine and the control group received saline. Progenies (n=30 per group) from six groups of breeders were all challenged by a varIBDV-SK09. Another two groups of progenies (n=30 per group) from saline and commercial vaccine given breeders were kept as unchallenged controls. Virus load was determined in bursa of Fabricius (BF) in each progeny group to measure the effect of vaccine on virus replication. Our data concluded delayed virus replication and in the broiler progeny from varIBDV-SK09 vaccinated broiler breeders.

INTRODUCTION

Infectious Bursal Disease (IBD) is an immunosuppressive disease that occurs throughout the world. The etiological agent is Infectious Bursal Disease Virus (IBDV) which is grouped under the family *Birnaviridae*. Serologically, IBDV is divided into serotype 1 and serotype 2. Serotype 1 is pathogenic in poultry and is sub classified as classical, variant (varIBDV) and very virulent (vvIBDV) strains based on virulence(1).

The main circulating IBDV strain in North America is varIBDV, except for the incidence of vvIBDV in California (3). Even though varIBDV does not result in huge mortality as vvIBDV does, subclinical infections lead to severe immunosuppression. Consequently, infected birds become easily susceptible to secondary infection leading to considerable economic loss. Previously, we reported that varIBDV infection in 43% broiler chicken farms in Saskatchewan, Canada, which was associated with high mortality and higher condemnation and lower meat production. Hence, Saskatchewan has been losing approximately 3.9 million kilograms of meat per year. (7) Despite the huge cost in disease prevention, commercially available vaccines in Canada fail to provide full protection for broilers due to the antigenic difference between the vaccine and circulating varIBDV strains. (5) We isolated five main varIBDV strains from Western Canada, namely varIBDV-SK09, SK10, SK 11, SK12 and SK13. The most prevalent strain is varIBDV-SK09 (60%) which exhibited potential to be a vaccine candidate. (4)

A variety of vaccine strategies have been applied for prevention of IBD in poultry industry. For broiler vaccination, live attenuated, recombinant and immune complex (Icx) vaccine are practiced. For hyperimmunization of broiler breeders, live vaccine priming followed by boosting with inactivated vaccine is widely applied to ensure adequate transfer of neutralizing maternal antibody to broiler progeny. (8)

The concept of immune complex vaccination against IBDV was first proposed by Haddad et al., in 1994. (6) The vaccine was developed by combining live IBDV with virus specific antibody. The vaccine

provides protection for broiler chicks with reduced bursal damage and work in the presence of preexisting antibody.

Since existing commercial vaccines are not effective in preventing IBD caused by varIBDV in broilers, this study was carried out to develop effective breeder vaccines to control varIBDV in broilers via efficient transfer of neutralizing maternal antibodies.

MATERIALS AND METHODS

Vaccination Preparation and Experiment Groups. Virus used for broiler breeder vaccination (varIBDV-SK09) were propagated in specific pathogen free leghorns. Bursae were pooled and homogenized to obtain a virus stock with 40% (w/v) suspension in sterile saline (5). The virus stock was titrated by embryo infective dose 50 (EID_{50}). Broiler breeders (n=20/group) were randomly allocated into five groups [group 1 = saline; group 2 = live varIBDV-SK09 (10 EID_{50}/bird); group 3 = inactivated varIBDV-SK09 (1 x 10^2 EID_{50}/bird); group 4 = live varIBDV-SK09 (10 EID_{50}/bird) + twice inactivated varIBDV (1 x 10^2 EID_{50}/bird/time point) and group 5 = immune complex varIBDV-SK09 ($2x10^2$ EID_{50} varIBDV SK09 + antisera with ELISA titer of 2000/bird)]. The virus was inactivated in 0.2% formalin solution (2). The immune complex vaccine was incubated at room temperature for 1 hour before administration. Broiler breeders were vaccinated at 17 weeks of age (live), 19 and 21 weeks of age (inactivated) and 27 weeks of age (immune complex).

To determine the efficacy of the varIBDV-SK09 vaccines, broiler progenies were hatched from each broiler breeder group (n=30/group). An additional group of commercial broiler chickens (n=30) was obtained to compare current vaccination programs in the field.

Broiler progeny were challenged with varIBDV-SK09 (10 EID50 per bird) orally at 6 days of age. Additional two progeny groups (n=30 per group) from saline vaccinated breeders and commercial breeders were not challenged and kept in a separate room as negative controls. Bursae were collected for viral load detection at 3, 6, 9, 13 and 29 days post challenge (dpc) (n=3/group/time point).

All animal experiments were approved by the University Committee on Animal Care and Supply, Animal Research Ethics Board, University of Saskatchewan.

Viral Load Detection. Total RNA from RNAlater (Invitrogen) stabilized bursa were purified with RNeasy Mini Kit (QIAGEN). RNA was reverse-transcribed with QuantiTect Reverse Transcription Kit (QIAGEN).

Bursal viral load was determined using real-time PCR (Mx3000P qPCR system, Agilent Technologies) and TaqMan probes. IBDV VP2 gene fragment was amplified using 5'-GGACACAGGGTCAGGGTCAAT-3' as forward primer and 5'-GCAGTGTGTAGTGAGCACCCA-3' as reverse primer. The TaqMan probe for VP2 was labelled with FAM and ZEN/IBFQ at 5' and 3', respectively. Chicken 18S RNA was targeted for normalization with a forward primer 5'-CGGCTACCACATCCAAGGAA-3' (18S-F) and a reverse primer 5'-GCTGGAATTACCGCGGCT-3' (18S-R). The TaqMan probe was labelled with HEX reporter dye at 5' and ZEN/IBFQ at 3'.

The IBDV VP2 and 18S RNA amplifications were performed in the same tube (20 µl total volume) using Prime Time-Gene Expression Master Mix (IDT) and 2 µl of cDNA template. The PCR amplification cycles were set as follows: initial melting at 95ºC for 10 min, 40 cycles of 95ºC for 15 seconds and 60 seconds at 60ºC. Serially diluted cDNA samples were used to determine PCR efficiency. The experiment was performed in triplicates with appropriate controls.. IBDV viral loads in bursal tissues were determined by ΔΔ Ct method. Negative Ct values were set to be 39.

RESULTS

IBDV viral load in two negative control progeny groups, i.e. progeny from saline and commercial vaccinated breeders without challenge, was undetectable throughout the experiment. Early and very high viral load was detected in progeny from saline treated breeders post varIBDV-SK09 challenge. The viral load in this group peaked at three dpc (24235 fold) and declined to a lower level at six dpc (3215 fold) and nine dpc (1277 fold), later reached undetectable level by 13 dpc. Although the virus was not detected at three dpc and 6 dpc in the progeny of broiler breeders that were vaccinated with commercial vaccine, the viral load expanded to 1580 fold, and 44129 fold later on nine dpc and 13 dpc, respectively. No virus was detected after challenge in the progeny from live breeder vaccine group at all time points studied during our experiment.

DISCUSSION

Maternal antibodies play an essential role in neutralization IBDV in commercial broilers during neonatal life and prevent the development of immunosuppression and subsequent opportunistic infections. Nevertheless, it has been indicated that maternal antibodies derived from commercially available IBDV vaccines rarely protect against

varIBDVs circulating in Canadian broiler chicken industry. Current study developed vaccines based on varIBDV-SK09 and evaluated their protective efficacy by measuring the virus load in the bursal tissue of progeny post homologous virus challenge. Meanwhile, we paralleled broiler chickens with breeder commercial vaccine and tested its protection.

Our study showed that virus replication in the bursal tissue from progenies of varIBDV-SK09 vaccinated broiler breeders delayed for various length of time depending upon the type of vaccine. The progeny of broiler breeders that were vaccinated with live varIBDV-SK09 vaccine had no virus replication detected at any of the time points (i.e., 3, 6, 9, 13 and 29 dpc). Our study showed that progeny from commercially vaccinated breeders delayed the vaccine replication for only 6 days after challenge. Overall this study revealed that var IBDV-SK09 breeder vaccine more effectively prevented virus replication in the bursa of broiler chickens compared to the commercial vaccine.

REFERENCES

1. Berg, T. P. V. D. Acute infectious bursal disease in poultry: A review. Avian Pathol. 29: 175–194. 2000.

2. Habib, M., I. Hussain, W. H. Fang, Z. I. Rajput, Z. Z. Yang, and H. Irshad. Inactivation of infectious bursal disease virus by binary ethylenimine and formalin. J. Zhejiang Univ. Sci. B 7: 320–323. 2006.

3. Jackwood, D. J., S. E. Sommer-Wagner, A. S. T. Stoute, P. R. Woolcock, B. M. Crossley, S. K. Hietala, and B. R. Charlton. Characteristics of a very virulent infectious bursal disease virus from California. Avian Dis. 53: 592–600. 2009.

4. Kurukulasuriya, S., K. A. Ahmed, D. Ojkic, T. Gunawardana, K. Goonewardene, A. Gupta, B. Chow-Lockerbie, S. Popowich, P. Willson, S. K. Tikoo, and S. Gomis. Modified live infectious bursal disease virus (IBDV) vaccine delays infection of neonatal broiler chickens with variant IBDV compared to turkey herpesvirus (HVT)-IBDV vectored vaccine. Vaccine 35: 882–888. 2017.

5. Kurukulsuriya, S., K. A. Ahmed, D. Ojkic, T. Gunawardana, A. Gupta, K. Goonewardene, R. Karunaratne, S. Popowich, P. Willson, S. K. Tikoo, and S. Gomis. Circulating strains of variant infectious bursal disease virus may pose a challenge for antibiotic-free chicken farming in Canada. Res. Vet. Sci. 108: 54–59. 2016.

6. Whitfill, C. E., E. E. Haddad, C. A. Ricks, J. K. Skeeles, L. A. Newberry, J. N. Beasley, P. D. Andrews, J. A. Thoma, and P. S. Wakenell. Determination of optimum formulation of a novel infectious bursal disease virus (IBDV) vaccine constructed by mixing bursal disease antibody with IBDV. Avian Dis. 39: 687–699. 1995.

7. Zachar, T., S. Popowich, B. Goodhope, T. Knezacek, D. Ojkic, P. Willson, K. A. Ahmed, and S. Gomis. A 5-year study of the incidence and economic impact of variant infectious bursal disease viruses on broiler production in Saskatchewan, Canada. Can. J. Vet. Res. Rev. Can. Rech. Veterinaire 80: 255–261. 2016.

8. Terrestrial Manual: OIE - World Organisation for Animal Health.

FIELD EXPERIENCES WITH PROBIOTICS

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Boehringer Ingelheim

SUMMARY

In 2017 Novozymes and Boehringer Ingelheim entered a joint distribution agreement with the goal of finding alternatives to antibiotic use in poultry production. FloraMax® a product that Novozymes acquired with their recent purchase of Pacific Vet Group was the first product on which we collaborated. FloraMax has been used in the market place for the past several years and this presented some unique opportunities since the product has a history. Therefore, our initial goal with FloraMax was to be certain we understood the basics of how best to use the product.

Several studies were conducted to evaluate how to properly handle, prepare, deliver and maintain the product while it is being administered on the farm. These results will be presented.

We will also discuss different programs that we explored focusing on what we believe would lead to best compliance. As is typical with live culture products it appears repeated usage is what leads to their success. The pros and cons of these different programs will also be presented.

Since FloraMax is delivered through the drinking water, grower understanding is a major factor that we will also discuss.

All these studies are ongoing and as our understanding further develops, more information will be presented.

EFFECTIVENESS OF VACCINATION OF ONE DAY-OLD BROILERS IMMUNIZED WITH A *CLOSTRIDIUM PERFRINGENS* TOXOID

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[B]Southern Poultry Research Group Inc

SUMMARY

*Clostridium perfringens (*C.P.) is often isolated from poultry feed and/or the poultry house environment. Since C.P. can be normal bacteria of the broiler intestinal tract, it is commonly found in the litter. The broiler disease, necrotic enteritis (N.E.), is a result of broiler intestinal damage usually occurring from *E. maxima* and the presence of a toxigenic C.P. strain. Currently, the poultry industry is experiencing an increase in N.E as a result of banning the use of growth-promoter antibiotics by some countries and/or current consumer preferences for decreased antibiotic use in food production.

A C.P. toxoid has been shown to transfer maternal antibodies to C.P. alpha toxin. These maternal antibodies have been shown to reduce the necrotic enteritis in the broiler offspring. The second strategy in this study was to vaccinate broilers subcutaneously (S.Q.) at one day of age with a partial dose of the toxoid, with or without the toxoid's maternal antibodies. Two breeder flocks and two hatchery sources were used for the chicks involved in this study.

The ability of the maternal antibodies from the C.P. toxoid vaccinated hens and/or the toxoid applied to broilers at one day of age, to reduce the negative effects of a toxogenic C.P. to broiler offspring in a necrotic enteritis challenge model, was evaluated through observation and recording of performance parameters among the different treatment groups.

The objective of this study was to determine the ability of the maternal antibodies from the C.P. toxoid vaccinated hens and/or the toxoid applied to broilers at one day of age, to reduce the negative effects of a toxogenic C.P. to broiler offspring in a necrotic enteritis challenge model.

MATERIALS AND METHODS

Animals. The study was performed at the Southern Poultry Research Group facilities, located in Athens, GA. Three thousand day-of-hatch straight run broiler chicks were obtained from a broiler industry cooperator. One thousand two-hundred were from a flock of hens that had been vaccinated by the broiler company with the C.P toxoid. Eighteen-hundred broilers were from a different flock. Birds were of the same breed and age-matched to the C.P toxoid vaccinated hens. All birds were vaccinated by spray cabinet with a coccidiosis vaccine at recommended dosage by the manufacturer. Only healthy appearing chicks were used in this study.

Housing. Upon arrival, chicks were raised in 5 x 10 feet floor pens (stocking density of 1.0 $feet^2$ per bird) with approximately four (4) inches of fresh pine shavings (at placement), in asolid-sided barn, with dirt floors under ambient humidity. Litter was not replaced or amended during the course of this study. Feed and water were available ad libitum throughout the trial. Each pen contained 1 (one) tube feeder and 1 (one) bell drinker (50 bird to feeder/drinker ratio).Thermostatically controlled gas heaters were the primary heat source for the barn (if needed).

One (1) heat lamp per pen provided supplemental heat during brooding. Fans were used to cool birds. Birds were provided a lighting program as per the primary breeder recommendations.

EXPERIMENTAL DESIGN

Three thousand birds were assigned to five treatment groups with 12 replicate pens per treatment and 50 birds per pen. Five hundred birds were assigned to each treatment group with 12 replicate pens per treatment group and 50 birds per pen. Pen facility was divided into 12 blocks with each block containing each of the five treatment groups. Treatment groups were assigned to pens using randomized complete block. Southern Poultry Research Group completed randomization and assignment of treatment groups to pens using Random Permutation Tables (1). The study began when birds were placed (day-of-hatch; DOT 0), at which time birds were allocated to experimental pens. Only healthy birds were selected. On DOT 0, group body weights were recorded by pen. No birds were replaced during the study.

RESULTS

As expected, the non-challenged control broilers had the lowest feed conversion (FCR) and heaviest body weight (BW) at 42 days. The mortality trend was similar in the N.E. lesion scores, due to chick quality issues faced at the beginning of the study. There was no significant reduction in N.E. lesions from the challenge control by any of the treatments. It appeared that the toxoid vaccinated hens broiler offspring did not demonstrate any reduction in N.E. mortality or N.E. lesions. The toxoid applied S.Q. in broilers, may have provided a slight benefit in reducing clinical N.E. Results for the C.P toxoid vaccinated hens may be somewhat confounded by early chick mortality from the hatchery.

ACKNOWLEDGEMENTS

We would like to thank and acknowledge the kindness and effort of the producers who collaborated with the subjects of study. In addition, we want to express our appreciation to the South Poultry Research Group Inc. (SPRGroup Inc), team and Dr. Charles Hofacre for the valuable collaboration on designing and performing the study.

REFERENCES

1. Cochran, W. G., and G. M. Cox. 1992. Experimental Design. 2nd Ed. John Wiley & Sons, New York, NY. Pg 582-583.

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

Table 1. Clinical necrotic enteritis assessment.

Treatment	Percent Mortality	Percent NE Mortality	NE Lesion
1. Non-Challenge Control	**6.333C**	**0.000C**	**0.000C**
2. Challenge Control	**21.000B**	**9.833B**	**0.408B**
3. Netvax Broilers	**28.500A**	**21.833A**	**0.925A**
4. Netvax Broilers plus S.Q. Netvax	**29.833A**	**22.833A**	**0.700AB**
5. Netvax S.Q. 1 time at Day 1	**19.667B**	**11.333B**	**0.483B**

INFECTIOUS CORYZA IN BROILERS IN THE CENTRAL VALLEY OF CALIFORNIA, PRODUCTION AND ECONOMIC IMPACT

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ABSTRACT

Infectious coryza caused by the bacterial agent, *Avibacterium paragallinarum*, not typically effecting broiler chickens, is diagnosed on a commercial broiler operation in the Central Valley of California in March 2017. The index farm had 300,000 chicks placed throughout 14 houses. First sign of clinical disease was detected at 19 days of age in one house. Clinical signs consisted of nasal and ocular discharge, cough, rales, and snicks. As the disease progressed, swollen, edematous heads and ruffled feathers developed as well. By 22 days of age, spread of the disease was noted, and by the time the birds reached 41 days of age, the entire index farm had contracted the disease. By processing, the farm experienced a staggering 24% mortality and despite treatment efforts along with increased biosecurity control, this disease was not contained on the index farm. Coryza spread across the four farm complex in a period of a few weeks.

Diagnostics were carried out through the CAHFS laboratory in Turlock, CA. Confirmation of the agent was done by bacterial culture, isolation, and PCR methods. Concurrent disease was not detected on the index farm. Serology and PCR for infectious bronchitis, avian influenza, and *Mycoplasma* were all negative. At processing, whole body condemn reached over 50%, and the remaining processed birds had disease involvement mainly consisting of airsacculitis at 97% of the flock. Feed conversion for this flock was 2.38. Comparing to a farm that processed the same week, located 6.5 miles away with WB 1.29%, disease involvement at 7.8%, and FCR at 1.75, it is clear coryza has the potential for causing detrimental impacts on broiler production.

This case demonstrates the important production and economic impact uncomplicated infectious coryza can have on meat-type chickens. It also raises the question about where this bacterium came from and how it was able to enter the farm and the possible modes of transmission from house to house, as well as from farm to farm on the complex.

INTRODUCTION

Infectious coryza is an acute respiratory disease caused by the bacterial agent, *Avibacterium paragallinarum*. *Avibacterium paragallinarum* is a pleomorphic rod shaped gram-negative bacterium. *A. paragallinarum*, historically known as *Haemophilus paragallinarum*, was first isolated in 1932, by De Blieck (5). This bacterium is challenging to culture and isolate and requires codependency from *Staphylococcus* when using blood agar media. The molecular techniques of PCR, which is both highly sensitive and specific, can be used to confirm the presence of *A. paragallinarum* (1).

Infectious coryza is often complicated by other respiratory pathogens, including mycoplasma, *E. coli*, infectious bronchitis, and/or fowl pox making isolation of the bacteria challenging (6). This disease is more commonly known for infecting layer chickens and birds that are raised on multi-age facilities in developed countries like the United States (6). However, there have been documented cases of coryza in meat-type birds in California and Alabama (5).

Here I will discuss a case study of uncomplicated infectious coryza infecting an operation of broiler chickens in the central valley of California that was diagnosed by the California Animal Health and Food Safety Laboratory System (CAHFS), Turlock location. This case study highlights the importance of rapid diagnosis through field findings and laboratory support, treatment, and prevention measures in controlling infectious coryza. Furthermore, this case study will discuss gross pathology and histological findings that were associated with the disease outbreak.

MATERIAL AND METHODS

Case history. This case involved four broiler farms over a course of three weeks. The index farm, G4, had 303,000 chicks placed and spread over 14 houses at approximately 21,600 per house. One house showed respiratory signs at 19 days of age. These clinical signs consisted of nasal and ocular discharge,

cough, rales, and snicks. By the following day, birds from the index house were reported to have swollen, edematous heads and ruffled feathers. A sample of these birds was submitted to the CAHFS laboratory in Turlock, CA for diagnostics.

Prior to initial observation of clinical signs by the farm laborers, mortality held at approximately 0.003% daily. At 19 days, mortality jumped to 0.1%. The following days mortality increased to 0.25%. By day 6, mortality had increased to 1.1%. Mortality in this house spiked to a daily total of 3.4% during the remaining course of the production cycle.

Spread of the disease from the index house was first noted at age 22, three days after initial observations. By the time birds were 41 days of age, all houses had acute respiratory disease and significant increase in mortality. Birds were processed at an average age of 45.29 days.

Field observations. Clinical sings progressed to swollen heads, conjunctivitis, catarrhal mucus membranes of the nasal concha, exudative to purulent sinusitis, torticollis, rales, sneezing, lacrimation, rhinitis, diarrhea, and lethargy. As the disease progressed extensive airsacculitis became prevalent as well. The neurologic signs of torticollis and circling were only observed in the most severely affected houses, that is, those that experienced the highest morbidity and mortality. In all affected houses, normal bird activity and sounds were subdued.

Laboratory procedures and results. The CAHFS laboratory cultured infraorbital sinus, lung, brain, airsac, heart, liver, and joint from submitted birds. The swabs from these tissues were placed on blood agar as well as chocolate blood agar. Both plates were cross-streaked with *Staphylococcus aureus* nurse cultures. The plates were incubated at 37ºC and 7% Co2 and examined at 24 and 48 hours post-incubation. Bacterial colonies compatible with *A. paragallinarum* were confirmed by a PCR specific for this microorganism (2). The most consistent histological lesions of the nasal cavity included accumulation of fibrino-heterophilic exudate within the infraorbital sinuses along with multifocal heterophilic infiltration of the mucosa of the nasal passages and multifocal mononuclear infiltration of the lamina propria of nasal passages. Eyelids were also processed for histology with the following findings noted, multifocal heterophilic infiltration of the conjunctiva; hyperkeratosis and multifocal coagulative necrosis associated with bacterial colonies, sometimes extending to the feather follicles. Grossly, birds showing neurological signs exhibited thick exudative material deep into the inner ear and extending to the air spaces of the cranial bones.

Treatment. Preliminary laboratory results were received when the birds were 27 days of age that were confirmatory of *Avibacterium paragallinarum* through use of PCR from culture isolates taken from sinuses of affected birds. Antibiotic administration began the following day for the entire ranch with water soluble Aureomycin at 25mg/lb, administered daily, for a course of five days. During the five day treatment course other houses on the farm showed similar clinical signs of rhinitis, increased lacrimation, and swollen heads. This treatment plan was repeated as clinical signs, mortality, and spread between houses continued. A medication withdrawal period of a minimum of 24 hours was observed prior to processing.

RESULTS

Despite the extended course of treatment, the closing flock mortality for G4 was a staggering 24.66%, with the majority occurring during the sixth and seventh week of life of the flock. Infectious coryza was not successfully contained at the index farm. During the three week ordeal, it was detected and confirmed at the surrounding farms at G1, G3, and G5. The age spread between the different farms was no greater than four days. Distance between these farms ranged from 300 to 1600 ft from the index farm, G4.

At processing, G4 birds had a whole body condemnation of 54.44%. Disease involvement, which mainly consisted of salvageable airsacculitis, was 97.76% for this flock. Adjusted feed conversion was 2.38. When comparing to a farm located 6.5 miles away that processed in the same week: 1.29%, 7.88%, and 1.75 for WB condemnation, disease involvement, and adjusted FCR, respectively.

DISCUSSION

This case demonstrates the important economic impact that infectious coryza can have on meat type birds. With the high mortality and whole body condemn, G4 processed 104,200 birds of the original 303,560 birds placed on the farm. The combination of high mortality, feed conversion, condemnation rate, and process flow interference at the plant resulted in a million dollar loss for the integrator for this one farm. Not only does infectious coryza have the ability to cause significant loss in terms of birds and processing efficiency, it also has lasting affects after the birds are shipped out and processed. The increased downtime between flocks that is needed to ensure proper cleaning and disinfecting adds to the economic impact this disease has on broiler production. The reduced movement of personnel between farms and complexes limits the resources the integrator has available for labor, supervision, and technical services.

As Droual reported in a 1990s case of infectious coryza in broilers in California, this is a disease that is primarily reported in layers and multi-age facilities. Furthermore, cases that have involved broilers in the past have been associated with breaches in biosecurity or when broilers have been located in close proximity to a layer or replacement pullet facility (5). This case raises the concern of a breach in biosecurity as well. Many laborers worked on the G4 farm, as well as other farms owned by the broiler integrator. Being under contract or as an employee, no person is allowed to own outside birds or live in association with another individual that has contact with non-company own birds. Could it be possible that one of these laborers introduced infectious coryza to the farm? While performing a biosecurity audit on the farm, several bird nests were found along the outside of the houses as well as one inside the index house. Could these wild birds be a possible source of infection? Wild birds serving as reservoir hosts for *Avibacterium paragallinarum* has not been well documented.

Finally, transmission of the disease in this case also serves as a point for discussion. Biosecurity protocol on the farm is comprehensive, consisting of clean coveralls, protective gear including hair net, plastic boots, and covering rubber boots. Laborers are also required to wash their boots off prior to entering into the houses with a quaternary ammonia disinfectant. It is possible that airborne transmission occurred not only between the houses, but also between the farms that were sequentially infected.

REFERENCES

1. Blackall, P.J., and R. Yamamoto. Chapter 6, Infectious Coryza. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson, and W.M. Reed, eds. American Association of Avian Pathologists, Inc., Kennett Square, PA. . P-p. 29-34. 1998.

2. Chen X., J.K. Miflin, P. Zhang, and P.J. Blackall. Development and application of DNA probes and PCR tests for Haemophilus paragallinarum. Avian Dis. 40:398-407. 1996

3. De Blieck, L. 1932. A haemoglobinophilic bacterium as the cause of contagious catarrh of the fowl (Coryza infectiosa gallinarum).*Vet J* 88:9–13.

4. Disease of Poultry 12th edition (blackalland serioano) Chpt 20 pg 789-803.

5. Droual, AA . A. Bickford, B . R. charlton, G. L. cooper, and s. E. channing. Infectious Coryza in Meat Chickens in the San Joaquin Valley of California. AVIAN DISEASES3 4:1009-1016, 1990.

6. Poultry Pathology Manual (Blackall and Chen) pg 327-331.

BIOSECURITY PRINCIPLES, FUNDAMENTALS, AND STRUCTURE

R. Munoz

PRINCIPLES

First principle: Biosecurity components according to Dr. Butch Baker and Dr. Tim Snider. "Biosecurity" is a general term that includes three major aspects: bio-exclusion, the prevention of any outside agent from entering a production animal operation; bio-management, the activities implemented to prevent agents from spreading within a facility (including the use of vaccines); and bio-containment, the protocols that prevent bacteria and viruses from spreading outside of the facility, even when their presence is unknown. Bio-containment is probably the most difficult aspect to implement, and the one that is most often forgotten about. (Figure 1)

Second principle: Five components from Dr. Herbert Sinner. The first component of cleaning, according to Dr. Herbert Sinner, is the chemical action, which is the product being and its chemical effect on the surfaces and the environment used (i.e. detergents and disinfectants). The second is the time taken to clean, which allows the chemical to have more exposure to the surface being cleaned. The third is the mechanical action, or the way the product surface contact and penetration as well as the forces applied for its incorporation and distribution or coverage. The fourth component is the temperature of the water. Warm water will enhance the strength of surfactants, allowing the chemical and mechanical actions to be more effective in reducing the presence of biofilm. Finally, the quality of water used has an effect, depending on the water's own hardness and/or bacterial contamination. (Figure 2)

Third principle: Integration of biosecurity according to Dr. Gregory Martin from Pennsylvania State University. The final objective of a biosecurity program should be to achieve lower to none economical losses due to infectious disease exposure or spread within a population. The disease (s) to list as a priority will depend of the local/regional endemic status and risk factors associated to the operations. Biosecurity is integrated with three major components. The first is the network of physical barriers and other lines of separation defined based on work flow, production flow, animal ages, susceptibility risk assessment and animals in need of quarantine, etc. The second component is the range of cleaners, disinfectants and other products used to reduce any potential biological threat to the production facility. The third is the use of logic and common sense. We need to keep in mind the bigger picture regarding the final objective, which is not usually included in written protocols. Protocols are not always adapted to changing conditions when a production unit faces unique circumstances. (Figure 3)

FUNDAMENTALS

One of the biggest challenge to biosecurity is the presence of biofilm and organic matter. These could become the substrate for bacteria growth and the development of microbiological challenges. Biofilm is the number one risk because it cannot be seen. Biofilm accumulation becomes critical when it limits the effect that disinfectants have on a surface. Dry cleaning takes out dust and gross organic matter, and then a wet cleaning removes more organic matter and the biofilm. If the biofilm is allowed to remain, it negatively impacts the performance of disinfectants, allowing surfaces (such as farrowing crate panels and the plastic surfaces on hatcheries) to become future challenges.

Biofilm is a layer made of polymeric extracellular secretion and microorganisms that coats a surface. It occurs in an animal production environment due to, for instance, secretions accumulating on a matrix layer over time. It acts as a mechanical barrier that lessens the effectiveness of disinfectants. Acid cleaners have the ability to penetrate the layer of slime, so disinfectants could be more efficient and demonstrate better effect on surfaces that have been prepared for disinfection.

Cleaning is the most important step. Before disinfection, we need to do the best dry and wet cleaning in order to reduce 90% of organic contamination and biofilm that could lessen the impact of any disinfectant. The difference between sanitation and disinfection results over an inner hard surface is the amount of bacteria or viruses reduction that one could achieve. These results are expressed in logarithmic (logs) values. Sanitation reduces microbiological material by 99% (2 logs), and disinfection reduces it by 99.99% (4 logs). After a good dry and wet cleaning, we finalize the process with a disinfectant in order to achieve the best decontamination possible in animal production facilities.

Another one of the biggest challenges comes from the surfaces types being cleaned. Some surfaces are more continuous, with less porosity and are more easily affected by cleaners and disinfectants. Another

challenge is presented by water with a high content of minerals, such as manganese, iron and calcium, adding deposits of salt. The mineral content of the water may interact negatively with the chemistry of products. Yet another challenge comes with the delivery of the cleaning product itself: proper equipment and correct pressure. These aspects and others, such as droplet size and coverage area, are easy to evaluate and to adjust as needed. Besides using a suitable cleaning product, we need to have a reliable mechanical program in place: one that uses the appropriate equipment and trained personal to facilitate the incorporation of the chemistry into the surface.

Viruses and bacteria have different levels of sensitivity or resistance to cleaners and disinfectants. Viruses with lipid envelopes are more sensitive, because they can be destroyed with the action of their surfactants of their detergents. Gram-positive bacteria are more sensitive than gram-negative bacteria, which have a structure that is naturally more resistant to disinfectants. Endospores and mycobacteria are also more resistant.

Peptidoglycan is one of the wall cell components of bacteria. This component is easier to affect by disinfectants. Gram-positive bacteria are more easily destroyed because 90% of their dry weight of its cell wall is peptidoglycan, compared with gram-negative bacteria which is just 10%. Disinfection is easier against gram-positive bacteria.

Viruses can have two different types of external structures. Some are lipid, which means they are more susceptible to disinfectants. Other viruses are considered "naked" and are more resistant. One example comes from viruses in swine and poultry that are more resistant, like circovirus. These viruses become more prevalent in production systems.

The Environmental Protective Agency (EPA) is the organization that regulates and sets disinfection standards for bacteria. If you want to have any claim of activity that you are trying to address with a disinfectant, you need to achieve a reduction of 6 log of a specific bacteria. For viruses, the number needs to be 4 log, and the number is a minimum of 3 log for cystic forms.

STRUCTURE

We have addressed the surfaces of the entire production facility, but we should not forget about water lines inside the buildings, which are sometimes more than 10-15 years old. Inside the waterlines can be found the extra challenge of biofilm and other accumulation that can affect the quality and taste of water. This potentially reduces optimum water consumption in animals. When water consumption is optimal, it assures better feed conversion for the flock/herd. Waterline accumulation, and the byproducts formed by it, also pose a risk because they can lead to bacterial contamination in the farm and also the pressure for water access.

Water intake is an important part of production. We need to remember that production animals should drink water at least twice the volume of what the feed they eat. If access to water is limited on a farm because of the quality of the water's taste or the bacteria contamination the animals' feed conversion is affected, limiting genetic production. If waterlines are not cleaned appropriately, it may limit production performance because of potential bacteria contamination. Biosecurity does not stop at visible hard surfaces, but continues into water lines that are not disinfected by application of standard surface disinfectants.

When we talk about taking proper steps to clean, we need to talk about the first five steps in the process of C&D. The cleaning section is the first major critical component. We dry clean to remove organic matter and minimize dust. Then, before applying detergent, we presoak the surface. Without the appropriate level of surface humidity, we cannot obtain better efficacy of the wetting agents in the detergent formulation. We never go into the shower and apply soap and shampoo before turning the water on, so the same common sense and logic applies. When cleaning, we need to presoak and then descale, taking out the accumulation of the scale.

The next step is to rinse and remove the detergent. Sometimes an additional rinsing and second application of detergent are necessary (combining acid first, then alkaline). After that, the next is to dry the surface, allowing for a natural air dry, so that the surface does not retain any water that can affect the dilution of the disinfectant; and final step is to disinfect. The heavy work of cleaning has been done first, removing at least 90% of contamination. Disinfection is important, but it is only the cap on the entire C&D process.

A quick hygiene evaluation of any critical surfaces, with the purpose of verifying how well the cleaning process was performed, is possible with the use of AccuPoint Advanced to measure adenosine triphosphate (ATP). Before applying disinfectant, you can use AccuPoint Advanced to evaluate how well the surface is prepared for disinfection, allowing for the maximum virus and bacteria elimination; efficacy of cleaning.

Monitoring and verification have become one of the key elements to move biosecurity programs from a descriptive format to a more systematic program. In such a program, protocols could generate metrics rather than just qualitative-descriptive assessments. As other farm processes such as vaccinations and

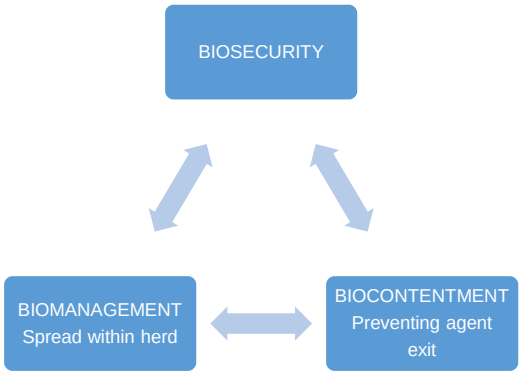
intestinal integrity are evaluated, it is necessary and critical to start implementing numbers behind biosecurity protocols that could be evaluated in a continuous way.

Consistent levels of main active ingredients in products are something that should be routinely verified to evaluate average/range of chemical concentration through pH strips. Qualitative or semi-quantitative tests could help to achieve optimum dilutions and approximate end product in PPM at use.

There are different type of C&D programs. The ideal is All In All Out (AIAO), in this type of animal production system once the production cycle finish, all the animals are removed from the facility. In this way it is possible to remove all the gross of the organic matter (dry cleaning) and proceed to the wet cleaning and disinfection. Following these steps, the organic matter and the biofilm will not interfered with the efficacy of the disinfectants. However, that is not always possible, so there are different approaches such as Semi-All in All Out for a building, a barn or AIAO room or aerospace. The success of these different scenarios depends on the definition of production flows, people/equipment flow, animal age, etc. (Figure 4)

Animal production managers could structure the internal lines of separation according to the above criteria and the support of biosecurity tools such as the Danish bench, foot baths, etc. To be able to reduce and eliminate the potential infectious disease exposure and/or spread within the production site. It is difficult, but it is not impossible, and we can do it by line up very descriptive protocols and perform monitoring of those protocols.

Figure 1.



REFERENCES

http://www.nationalhogfarmer.com/health-diseases/0615-building-functional-biosecurity-barriers Dr Butch Baker (Joe Vansickle)

http://porkgateway.org/resource/biosecurity-of-pigs-and-farm-security/ Dr Butch Baker

https://www.pig333.com/tips_on_pigs/traffic-control-for-swine-production-sites_8815/ Dr Tim Snider

http://www.wattagnet.com/articles/27885-poultry-farm-biosecurity-principles-you-should-know Dr Gregory Martin

http://ethanolproducer.com/articles/10252/ Dr Herbert Sinner

Figure 2.

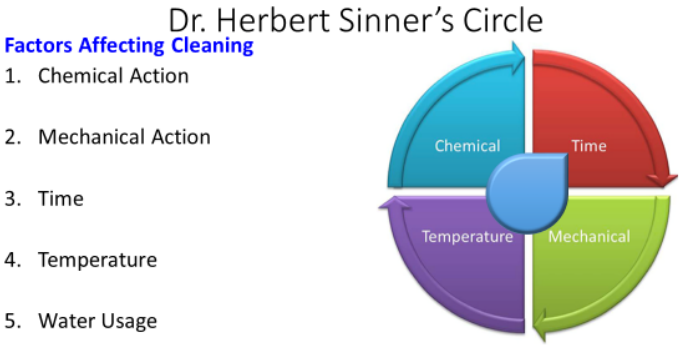


Figure 3.

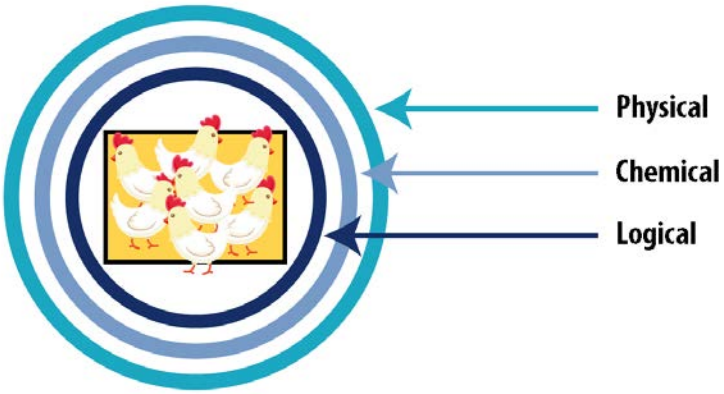
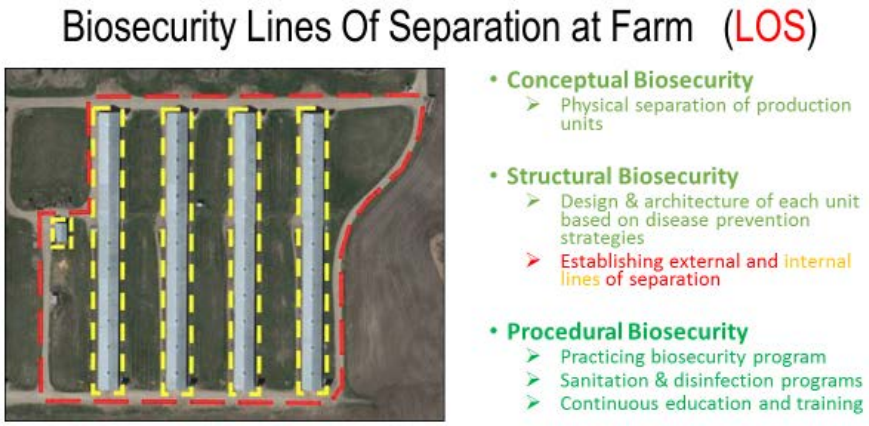


Figure 4.



EFFECTIVE THERAPEUTIC USE OF ENRAMYCIN TO REDUCE ROUTINE NON-THERAPEUTIC ANTIBIOTIC APPLICATION

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INTRODUCTION

Necrotic enteritis (NE), caused by *Clostridium perfringens*, Types A and C, has been associated with many contributing factors. One important factor often associated with NE is intestinal damage due to coccidiosis. Mild coccidiosis lesions may be associated with live coccidiosis vaccination, with a peak at day 18 to 24, while mild lesions may be associated with anticoccidial programs at about four weeks of age. Other factors that influence the occurrence of NE include climate, feeds containing non-starch polysaccharides, high protein feeds, feed form (fine or coarse grind) and abrupt feed changes. These can act in concert to increase the risk of NE during specific time periods in a broiler growout cycle, sometimes resulting in highly predictable NE outbreaks. For instance, the age at which NE occurs can be narrowed to timing as specific as 17 to 18 days of age in some coccidiosis vaccinated flocks, while feed change and subclinical coccidiasis may account for NE that typically occurs closer to four weeks of age. These are two windows when NE might occur with regularity, particularly when antibiotic growth promoters (AGPs) with anti-clostridium activity are not routinely employed, and particularly during specific seasonal or climatic conditions.

NE may also occur unpredictably, with underlying conditions and timing unique to each outbreak.

These studies investigated the use of enramycin as a therapeutic antibiotic under two infectious, challenge conditions. Enramycin was administered two days prior to an expected onset of NE and, alternatively, was administered as treatment after clinical signs of NE were observed, as might be necessary in an unpredictable outbreak. Investigators also measured tissue residues to determine appropriate withdrawal time for a dose of 20 ppm used for 10 days.

STUDY DESIGN

Study 1: Therapeutic use of Enramycin. Male Ross broiler chickens from the same hatch group and breeder flock were sourced from a commercial hatchery in Canada. A total of 665 chicks were divided into seven blocks of five contiguous treatment group pens, each containing 19 birds. A randomization method was used to assign treatments to each pen within a block.

All of the pens received the same wheat-based, non-medicated broiler starter diet until the assigned treatment commenced. All challenged groups were challenged via feed on day 14 with CP33, an isolate from an NE outbreak in commercial broilers in Ontario, Canada. Following challenge, pens were given a non-medicated grower ration except Group 3 (see treatment groups).

The treatment groups in this study consisted of Group 1- non-medicated, unchallenged controls, Group 2 - non-medicated, challenged controls, Group 3 - challenged birds treated with 20 ppm enramycin from day 12 to day 21 (treatment started before challenge), Group 4 - challenged birds treated with 20 ppm enramycin at the first sign of clinical NE and Group 5 - challenged birds treated with 30 ppm enramycin at the first sign of clinical NE. Groups 4 and 5 began treatment on day 17 and continued for seven days to day 24. After treatment, all groups were placed on non-medication rations until the study terminated on day 28.

Study 2: Tissue residue depletion. Cobb 500 birds (33 male and 33 female) were selected at 25 days of age after being reared from day one on non-medicated feed. Birds were acclimatized until day 32 in pens of five birds each by sex (12 total) with two pens containing three birds each (reserve group for quality control). Birds were treated with a target treatment of 25 ppm enramycin (actual determined value was 23.4 ppm in final feed) for a total of 10 days. After 10 days, birds were sacrificed for determination of residues in muscle, skin-with-attached fat, liver, and kidney samples.

RESULTS AND DISCUSSION

Study 1: Therapeutic Use of Enramycin. The NE challenge model produced 31.8% mortality in the non-medicated, challenged control birds from day 14 to day 28, significantly more than the unchallenged control mortality of 2.4% during the same time period. Birds treated with 20 ppm enramycin beginning two days prior to challenge had 5.0% mortality, not significantly different compared to unchallenged control birds, but was significantly different from the non-medicated, challenged control ($P < 0.05$). When treatment was held until the first clinical signs were noted, a numerical reduction in mortality (20 ppm – 19.6 % and 30 ppm – 23.3%) was noted when compared to non-medicated, challenged controls. There was no advantage to using the higher antibiotic inclusion rate.

These results underscore the importance of understanding necrotic enteritis infection dynamics when enramycin will be used as a therapeutic drug. Treatment just prior to the typical appearance of clinical signs will have greater efficacy compared to treatment beginning after the onset of clinical signs and mortality.

Study 2: Tissue residue depletion. A maximum residue limit (MRL) of 30 ppb has been established by the Japanese Ministry of Health. Residues in muscle and skin-with-attached fat were each below the limit of quantification for enramycin fractions A and B (4.60 ppb and 3.30 ppb, respectively). Residues in liver and kidney were an average of 19.4 ppb and 14.1 ppb respectively at zero hours' withdrawal. This supports a withdrawal time of zero days for poultry flocks treated with enramycin at 20 ppm in the feed for 10 days.

CONCLUSION

Enramycin may be used to reduce mortality due to NE when used at 20 ppm. The studies showed that under the experimental conditions where animals were observed and treated individually, the treatment can be initiated either just before the typical onset of clinical signs or just after clinical signs appear. Under field conditions it is clear that when necrotic enteritis hit a poultry house, treatment will be initiated in the whole house at once at a time when some birds have already shown clinical signs and other not. Based on the clinical findings reported in this paper, the earlier the house treatment is initiated the more efficacious it will be. A withdrawal time of zero days has been established for flocks treated with enramycin at 20 ppm in the feed for 10 days.

(Full length articles with greater detail on the materials and methods used in these studies are under review at the *Journal of Applied Poultry Research*.)

REFERENCES

1. Songer, J.G., Clostridial enteric diseases of domestic animals. Clinical Microbiology Reviews, 1996. **9**(2): p. 216-234.

2. Opengart, K.a.J.G.S., Necrotic Enteritis, in *Diseases of Poultry*, J.R.G. D.E. Swayne, L.R. McDougald, L.K Nolan, D.L. Suarez, V. Nair, Editor. 2013, Wiley-Blackwell Publishing: Ames, Iowa. p. 949-953.

3. Al-Sheikhly, F.a.A.A.-S., Role of coccidia in the occurrence of necrotic enteritis of chickens. Avian Diseases, 1980. **24**(2): p. 324-333.

4. Paiva, D.a.A.M., *Necrotic enteritis: applications for the poultry industry*. Journal of Applied Poultry Research, 2014. **23**: p. 557-566.

5. Williams, R.B., Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology, 2005 **34**(3): p. 159-180.

6. Mathis, G. Coccidia, always present. 2009; Available from: www.wattagnet.com/articles/283-coccidia-always-present

7. Reyna, P.S., L.R. McDougald, G.F. Mathis, Survival of coccidia in poultry litter and reservoirs of infection. Avian Diseases, 1983. **27**(2): p. 464-473.

8. Chapman, H.D., J.R. Barta, M.A. Hafez, P. Matsler, T. Rathinam and M. Raccoursier, The epizootiology of Eimeria infections in commercial broiler chickens where anticoccidial drug programs were employed in six successive flocks to control coccidiosis. Poultry Science, 2016. **95**: p. 1774-1778.

9. Long, J.R., Necrotic enteritis in broiler chickens I. A review of the literature and the prevalence of the disease in Ontario. Canadian Journal of Comparative Medicine, 1973. **37**(3): p. 302-308.

10. Smith, J.A., Experiences with drug-free broiler production. Poultry Science, 2011. **90**: p. 2670-2678.

11. Timbermont, L., F. Haesbrouck, R. Ducatelle and F. Van Immerseel, Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology, 2011. **40**(4): p. 341-347.

12. Kaldhusdal, M., S.L Benestad, and A. Lovland, Epidemiologic aspects of necrotic 205 enteritis in broiler chickens - disease occurrence and production performance. Avian 206 Pathology, 2016. **45**(3): p. 271-274.

13. Necrotic enteritis and associated conditions in broiler chickens, in *Aviagen - RossTech*. 1999, Aviagen.

INFECTIOUS BRONCHITIS VIRUS INFECTION IN ONTARIO: AN UPDATE ON THE CHANGING STRAINS OVER TIME

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INTRODUCTION

Infectious bronchitis virus (IBV) infection of chickens in Ontario and Canada was for many years limited to sporadic outbreaks. Previously, a small subset of IBVs from Ontario were genotyped and these viruses were related to variant IBVs circulating in the US (1). Starting in early 2012 and in 2013 an increased number of IBV-associated cases have been reported (2). These cases involved respiratory disease, increased mortality, urate nephrosis and egg-production drops. The field situation shifted in 2016 and 2017 when IBV emerged as the most important viral chicken pathogen in Ontario generating a marked increase in submissions related to IBV infection (Figure 1). IBV infection caused severe disease and high losses affecting all commodity groups. The objective of this study was to genotype IBVs to characterize the shifting of the predominant IBV strains over time in samples submitted to the Animal Health Laboratory (AHL) at the University of Guelph in Ontario, Canada.

RESULTS

Based on sequence comparisons of the hypervariable region of the S gene from 505 IBVs, field strains detected in Canada from 2014-2017 could be divided into 5 major groups:

1) vaccine-like, classic viruses, such as Connecticut and Massachusetts
2)" indigenous" Canadian variants not described elsewhere, such as strain Qu_mv
3) variant viruses related to strains described in the US such as DMV/1639/11, California 1734/04, CU_82792/GA98 and Pennsylvania Wolg/98
4) Exotic, non-Canadian, non-US viruses, such as strain 4/91 (793b)
5) "untypable" IBVs not showing significant similarity to previously described IBV strains (Table 1).

DISCUSSION

Massachusetts-type vaccines are the primary IBV vaccine type used in Canada, occasionally used in combination with Connecticut -derived strains. These vaccines can provide cross-protection against various IBV variants, presumably due to cross-reactivity involving cytotoxic T lymphocytes. However, recent incursions of 4/91, California 1734/04 and DMV/1639/11 -like viruses were associated with various and often severe disease processes in all chicken commodity groups. It appears that challenge with these "new" viruses cannot be controlled by vaccines currently available in Canada and alternative vaccination protocols are being considered.

REFERENCES

1. Ojkić D, Swinton J, Binnington B. Phylogenetic analysis of Ontario infectious bronchitis virus isolates. 51st Western Poultry Disease Conference, Puerto Vallarta, Mexico, April 30-May 4, 2002.

2. Martin EA, Brash ML, Hoyland SK, Coventry JM, Sandrock C, Guerin MT, Ojkić D. Genotyping of infectious bronchitis viruses identified in Canada between 2000 and 2013. Avian Pathol. 2014 Jun;43(3):264-8.

Figure 1. Number of samples submitted for testing by IBV PCR from 2012-2017.

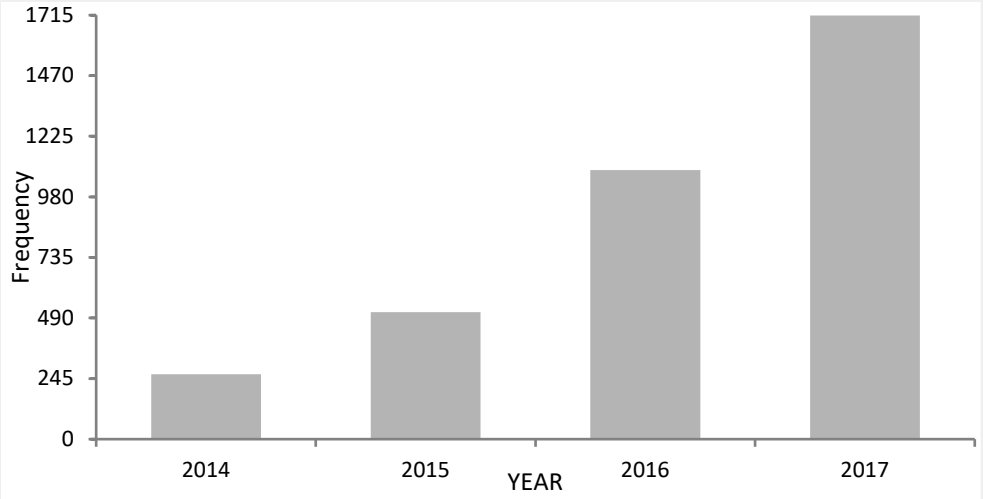

Table 1. Summary of incidence of IBV strains genotyped from 2014-2017.

	STRAIN	2014	2015	2016	2017
1) Vaccine-like	**Mass**	42.86%	43.66%	31.54%	28.26%
	Conn	7.14%	25.35%	4.62%	6.52%
2) Indigenous Canadian	**Qu_mv**	0.00%	0.00%	3.85%	1.45%
3) US-variant	**CA 1737 04**	7.14%	14.08%	20.77%	9.42%
	CU82792	0.00%	4.23%	0.77%	1.45%
	DMV	0.00%	1.41%	23.85%	46.01%
	GA 2012	0.00%	4.23%	1.54%	0.36%
	PA_Wolg	0.00%	0.00%	2.31%	0.00%
4) Exotic	**4_91**	28.57%	5.63%	6.15%	2.90%
5) Untypable	**Not typed**	14.29%	1.41%	4.62%	3.62%

EFFECTS OF A WATER APPLIED BIOPROMOTOR AND FEED APPLIED MOS ON THE PREVALENCE OF COLIBACILLOSIS AND EGG PRODUCTION IN COMMERCIAL HEAVY BREEDERS

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ABSTRACT

Commercial poultry production farms are under increasing pressure to reduce the use of antibiotics while maintaining health standards and pathogen control. Currently, there is an increased need for the development of effective products that serve as alternatives to antibiotics. We have developed a water applied biopromoter through careful selection of different types of prebiotics strategies (inactivated fermented *Bacillus subtilis* fragments and yeast cell wall extracts) that improves mucosal integrity and controls gram negative pathogens, while increasing productivity parameters and health status of the birds.

A commercial poultry farm (Ross 508 heavy breeders), Cuernavaca, Mexico, with a historical problem of increased mortality, increased diarrhea and reduced egg production attributed to Colibacillosis beginning at week 28 was divided into two treatment groups (n=2 houses). House 1 (n=20000 heavy breeders) served as the untreated control and was fed a commercial basal diet without AGP and House 2 (n=20000 heavy breeders) was treated with two doses of the biopromoter (0.2mL/bird) in the drinking water at week 24 and 18 days later and MOS incorporated into the standard commercial basal diet (2kg/ton) free of AGP during weeks 25-34.

Results show significantly increased mortality in the untreated house beginning at 28 weeks and continuing until 34 weeks, comparable with historical farm data, while birds in the treated house presented normal mortality consistent with stress associated during the peak of production. Microbiological analysis confirmed that the increased mortality in the untreated house was consistent with Colibacillosis. Through peak of production the treated house achieved 84.48% production while the control house achieved a maximum production of 67.03%. Analysis further showed egg production in the treated house was significantly higher (n=899,230) when compared to the untreated control house (n= 497,425).

These data indicate that treatment with two doses of the biopromoter and temporary incorporation of MOS into the commercial basal diet can moderate the effects of *E. coli* in heavy breeders. Furthermore, this strategic approach of combining prebiotics provides an excellent alternative intervention strategy to chemotherapeutic agents.

INTRODUCTION

Colibacillosis is a term commonly used for infections caused by *E. coli* in animals. In birds, *E. coli* infection is considered secondary to other agents and the manifestation of the disease is extra intestinal. Colibacillosis is one of the major diseases of modern aviculture because of the great economic losses worldwide as colisepticemia, peritonitis, pneumonia, pleuropneumonia, aerossaculitis, pericarditis, cellulitis, coligranuloma, chronic respiratory diseases complicated, panophthalmia, salpingitis, head syndrome swollen, osteomyelitis, oophoritis and synovitis (Berchiere, 2009). The products used in this trial are detailed below.

The biopromoter is produced from the selection and enhancement of strains of saprophytic bacteria of the species Bacillus subtilis, which act by modulating innate immunity and inflammation, optimizes the innate immune response against different pathogens and reduces the negative effects of inflammation response. It also directly influences the integrity of mucous membranes, through the reinforcement of intercellular junctions (Tight Junctions) and acceleration of tissue repair, as well as indirectly in the prevention of mucosal wear caused by oxidation reactions resulting from the immune/inflammatory response.

The MOS is composed of organic acids (acetic, formic and propionic with the salts ammonium formate and ammonium propionate) inserted into mineral particles making it capable of acting in the feed and intestinal tract of birds. In addition, it has wall of yeast. This association of acids associated with mineral carrier provides the favorable bowel conditions for multiplication and colonization by acidophilic (acid-tolerant and producing bacteria). The use of protected organic acid in a feed reduces microbial contamination of the feed, controls pathogenic enterobacteria in the gastrointestinal tract such as *Salmonella* spp., *Escherichia coli* and *Clostridium* spp., favors colonization of the intestine by acidophilus microflora and has synergy with

probiotics and prebiotics of this nutrient uptake and improved egg quality (2). The Manicologosacarideos (MOS) presents as a product with ability to adhere to pathogenic bacteria (type 1 fimbriae), such as prebiotics of acidophilic bacteria (Lactobacillus sp.), Whereas Betaglucans (β1,3 and β1,6) present immunostimulatory characteristics.

MATERIALS AND METHODS

Two flocks of breeders of the Ross 308 genetic line were used in the region of Cuernavaca, Mexico. All birds receive the same diet and the same management of the farm. The animals were divided into two treatments of 19,000 birds each one. Treatment 1 called Control and Treatment 2 with biopromoter and MOS additives. Treatment 2 birds (T2) received the biopromoter at week 24 of age (0.2 mL per bird in drinking water) and a second dose 18 days after the first dose. These birds also received MOS in the feed (2 kg/ton) between the 25th and 34th weeks of age. The parameters measured were poultry weight, mortality, percentage of daily egg production, percentage of hatching eggs and dirty eggs.

RESULTS AND DISCUSSION

The flock with the proposed products started production of 2.26% with 25 weeks while the control lot presented a production of only 0.74% in the same week. The mean production for the treatment group was 62% in the period versus 39% in the control flock. The egg production peak was 84.48% in the treatment group and 67.03% in the control group. In absolute numbers the total eggs produced were 899,230 and 497,425 eggs per flock.

The weights of birds were better in the flock with the proposed additives, following the predicted weight of the line that the flock control even though both flock had similar weights at week 23. This is due to intestinal integrity allowing better nutrient uptake from ration.

The best indicator that treated birds are healthier and has less challenge is bird mortality. The data from the control lot correspond to the company's history, that is, an increase in mortality at week 28. The flock with biopromoter and MOS showed a stable and normal mortality at moments of stress during egg production peak.

The percentage of hatching eggs was higher in the flock with biopromoter and MOS when compared to the control flock. It is important to evaluate that in the period of higher egg production the additives provided better flock egg quality causing direct impact on the number of bird/Breeder. The difference between the lots reached 2% in the weeks of production peak. The number of hatching eggs was 386,226 more in the test lot. The number of dirty eggs was also lower in the test lot.

Laboratory analysis. Bacteriological and histopathological tests of intestine, liver, spleen and pancreas were doing at weeks 24 and 27 of age. In the first bacteriological test, *E. coli* was found in the pancreas and intestine and histopathological test found mild lymphocytic cholangitis related to *E. coli*. In the second sample, some birds presented in the bacteriological test the presence of *Staphylococcus* in the spleen and *E. coli* in the pancreas, while the histopathology revealed lymphocytic cholangitis and lymphocytic and heterophilic peritonitis caused by *E. coli*.

CONCLUSION

Due to primary factors that allowed the manifestation of colibacillosis in lots of the company that had increased mortality since the 28th week of age and persisted during peak production that failed to achieve satisfactory results. With the administration of biopromoter in drinking water and MOS in the feed the birds did not present the usual scenario improving the parameters such as mortality and egg production. The best intestinal health of the birds that receive the proposed additives was demonstrated in the number of hatching eggs, dirty eggs and birds weight. The laboratory results go against the reality of the farm.

REFERENCES

1. Berchieri J. A. et a l(2009): Doenças das aves. 2º Edição. FACTA. 457-471.

2. Barnes, H. J., Gross, W. B. Colibacillosis. Disease of poultry Necrotic enteritis. In: B. W. Calnek (ed.) Diseases of Poultry. 10th edition. Iowa State University Press, ed. Ames. 1997. p. 131–141

MONITORING MYCOTOXINS IN SAMPLES FROM SEVERAL REGIONS OF MEXICO

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ABSTRACT

The demand for quality raw materials for the production of food for animal production is always constant. The grains that are an important part of this production suffer from climatic effects such as temperature and humidity and even after harvesting can undergo changes due to storage. These changes include the production of mycotoxins derived from fungi present non-grainy and which cause diseases in animals including loss of zootechnical parameters. In order to demonstrate the mycotoxins present in raw materials and finished food in Mexico, different samples from different regions of the country were analyzed. Of the samples analyzed during the whole monitoring period, 77.25% were positive for one or more mycotoxins, with increased contamination by Afla (66.11%), followed by Fumo (34.4%) and Zea (34.4%). Also Ocratoxin was found in the samples with a high ammount.

INTRODUCTION

The secondary metabolites of some strains of contaminating fungal of grains can cause economic losses at all levels of production by having toxic effects when they are ingested by animals even at low levels of concentration since there is a greater risk of additive and synergistic toxic effect with the multiple presence of mycotoxins. It is known that mycotoxins are hepatotoxic, carcinogenic, neurotoxic, estrogenic and immunosuppressive. So it is preponderant to evaluate their presence routinely not only in the ingredients but also in the feed due to possible emerging contamination both in the feed meal and in the important final destination; the feeder of the animals (1).

MATERIALS AND METHODS

A total of 236 samples of ingredients for poultry and pigs such as white corn, yellow corn, sorghum, soybean, canola, wheat, corn bran and wheat bran, from North, Central and South Mexico were analyzed for mycotoxins in a 17-month period from January 2016 to May 2017. The mycotoxins analyzed were Aflatoxin (Afla), Fumonisin (Fumo), Deoxynivalenol (Don), T2, Ochratoxin (Ocra) and Zearalenone (Zea) using for this monitoring methodology the ELISA kit (Enzyme Linked Immunosorbent Assay) facilitating the fast obtaining of the results and with the possibility of carrying out the analysis in situ. Of the total samples analyzed in both periods, the samples of mash feed represented 73.73%, following the raw materials with 24.15% and the feed palletized with 2.12%. The samples analyzed in 2016 (Fig. 1) based on corn represented 84.6%, yellow corn 5% and sorghum 5% of the total samples, while for the period up to May 2017 the ingredients they represented 77%, 10% and 4%, respectively.

RESULTS AND DISCUSSION

Of the samples analyzed during the whole monitoring period, 77.25% were positive for one or more mycotoxins, with increased contamination by Afla (66.11%), followed by Fumo (34.4%) and Zea (34.4%). The prevalence of Afla, so far this year, is considerably increased in its detection in samples (64.29%) compared to its detection in 2016 (37.9%). In relation to Ocra, the same is observed in the average detected in the current year (15.88 PPB), in contrast to the 2016 average (6.82 PPB) (Fig 4). The LAMIC laboratory of the Federal University of Santa Maria (UFSM) in Brazil establishes a maximum of 500 PPB of Fumo for birds and adult pigs where an extremely high level (> 1,000 ppb) is observed in both periods (Fig. 1-2). The same happens with Ocra in the two periods where the average is above the established maximum level (5 PPB) for both adult species. The means obtained so far in 2017 from Don and Zea represent a great risk for pigs at all stages of production and for young birds (Figure 2), following the recommendations of LAMIC (2).

CONCLUSIONS

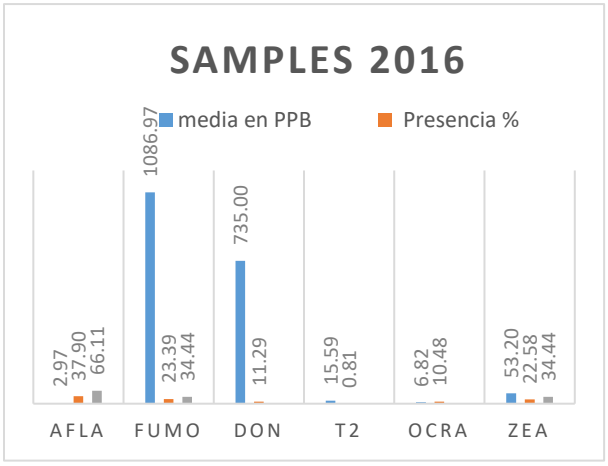
With this monitoring we could observe that Afla, Don and Zea had a considerable increase in their detection in samples. Ocra and Zea present the highest increase in their average level while Fumo and Don remain elevated in both periods. Quite the opposite happens with T2 where in the current period it has been decreasing.

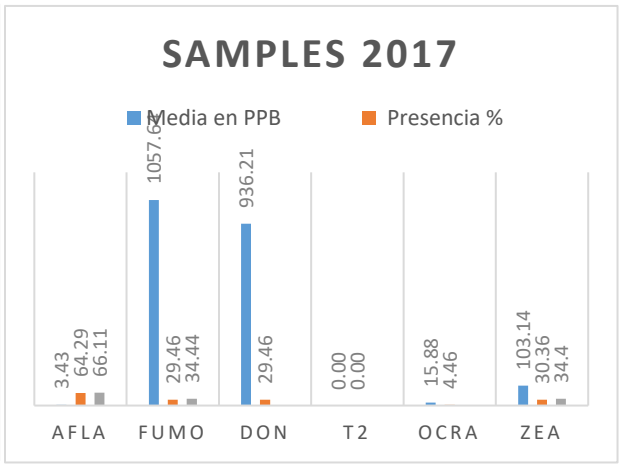
REFERENCES

1. poultryscience.org/A trend analysis of mycotoxin prevalence in the Americas from 2009 to 2013.

2. Mallman et al. Micotoxinas como gerenciar o riscos & minimizar o problema. Universidade Federal de Santa Maria (UFSM), Departamento de Medicina veterinaria Preventiva, (LAMIC) Avinews América Latina, Edición Brasil. marzo del 2017.

Figures 1 and 2.





UTILIZING MULTINOMIAL AND SPACE-TIME PERMUTATION MODELS TO UNDERSTAND THE EPIDEMIOLOGY OF INFECTIOUS BRONCHITIS IN CALIFORNIA BETWEEN 2008-2012

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ABSTRACT

While infectious bronchitis virus (IBV) has been described as one of the most economically important viral respiratory disease in poultry, there is little analyses of outbreaks utilizing spatial statistics. In order to better understand how the different genotypes of IBV behave spatially and temporally, GIS based mapping coupled with spatial and spatial-temporal statistics were used to identify statistically significant clustering of multiple strains of infectious bronchitis (IB) between 2008 and 2012 in California. Specifically, space-time permutation and multinomial models were used to identify spatial and spatial-temporal clusters of various genotypes of IBV. Multinomial models identified two statistically significant spatial-temporal clusters and one statistically significant spatial cluster. From a practical perspective, multinomial clustering approaches may be advantageous for studying IB because the model allows the different genotypes of IB to be independent nominal variables, thereby allowing for a more detailed spatial analysis. To that point, based on their risk ratios, the genotypes classified as vaccine were identified as the most significant contributor to two of the three mutinomial clusters.

INTRODUCTION

The complex ecology of IBV viruses in poultry producing areas involving vaccine types and variants and the subsequent generation of even more variants (23) is the main reason why the spatial and temporal behavior of IBV is so important to understand and study.

Currently an extensive amount of research has focused on variant types (3, 9-11, 13, 19, 20, 25), vaccinations protocols (1, 13, 17, 18) and the relationship between new IBV variants and the emergence of disease (6, 13). However, there are no studies that investigate how the different subtypes of IBV behaves spatially and/or temporally with respect to clustering and distribution. Using spatial-temporal statistical models to look at infectious diseases like IB can be an important tool in determining critical time-space boundaries with respect to IB prevention and control programs.

Among the tools available to explore questions regarding spatial-temporal disease distribution, the space-time permutation analyses and multinomial models have been used for retrospective disease cluster detection of different diseases (7, 15, 22). Briefly these models utilize, “scanning windows” which move across space (spatial only), time (temporal only) and space and time (spatial-temporal) in order to identify statistically significant clusters of disease. Specifically, the scanning window covers each possible time interval of each possible geographical location. Statistically significant clusters appear when a statistically significant number of premises are observed based on a log-likelihood ratio statistic (10). Additionally, the multinomial model allows for the independent analysis of different IB variants (14, 15) thus providing detailed information about the type(s) of IB that are statistically significant within a cluster.

The objective of this study was to use space-time permutation and multinomial models to identify statistically significant clusters of IBV in space, time and time and space in California.

MATERIAL AND METHODS

IBV cases. Out of 1,444 cases of IBV identified in California between 1997 and 2012, 131 IBV cases were identified with address information and were hence included in this study. Positive cases with address information were diagnosed by the California Animal Health and Food laboratory System (CAHFS) during the period between September 1 2008 and

December 31 2012.(8). The IBV genotypes identified in our study included California variant 99 (Cal 99), California variant 1737 (CA1737), Mass, and Conn. To represent the effects of vaccine related strains, Conn, and Mass were grouped in a single category named "vaccines" (Vacc). Cases without information regarding IBV stains or cases that did not match sequences in GenBank were classified as NA. Additional data collected that was linked to each IBV variant included; date of submission, IBV genotype and submitter.

Spatial and space-time analysis. In order to visualize the distribution of IBV cases in California during the study period, each positive case was spatially associated with the company performing the submission. All cases were mapped using this approach. To investigate IBV clusters in space and time a Space-Time Permutation Model (STPM) were performed using SaTScan™ version 9.1.1 (National Cancer Institute, Bethesda, Maryland, USA). To explore the spatial and spatial-temporal clusters in a single analysis at the genotype level a multinomial scan analysis was performed via SaTScan. The statistical significance of 'most likely clusters' (i.e. clusters that are least likely due to chance) in all the models fitted was evaluated through Monte Carlo simulations (999 replications) and p-values < 0.05 were considered statistically significant to reject the null hypothesis of random distribution in time or space.

RESULTS

Among the 131 IBV-related cases included in the study genotypic identification was performed on a total of 88 cases (67.2, 95% CI= 58.4-74.9) with a distribution as follows: 63 Cal 99 (48.1%, 95% CI=39.3-56.9), 4 CA1737 (3.1%, 95% CI=0.9- 8.1), 18 Conn (13.7%, 95% CI=8.5- 21.1), 3 Mass (2.3%, 95% CI= 0.6-7.1) and 43 NA (32.8%, 95% CI=25.02-41.6). 97% of the cases represented broilers (n=127, 95% CI= 91.8- 99) and 3% layers (n=4, 95% CI= 0.9-8.1).

The STPM identified 6 space and time clusters, including three that were statistically significant (p value<0.01). All three significant spatial-temporal clusters were detected at least in two time extents having durations of 131, 65 and 179 days and a radius of 15.78, 33.87 and 32.25 km respectively. The odds ratio (i.e. rate between observed and expected cases) among the significant clusters was 6.75, 4 and 2.27 respectively. Broiler producers were predominant in all the significant clusters obtained in the STPM analysis.

With respect to the identification of spatial-temporal clusters using the multinomial model, results showed significant clusters for different IBV types during the study period. Specifically, the spatial-temporal analysis showed two statistically significant clusters (p value<0.01). In the first cluster (i.e. cluster 1 from the spatial-temporal analysis), the category Vacc had the highest relative risk (2.68) within the different genotypes of IB. However, Cal99 (n = 7) was the most predominant in the cluster with respect to the total number of cases. In contrast, the second significant spatial-temporal cluster (i.e. cluster 2 from the spatial-temporal analysis) was conformed only by NA cases. With respect to the spatial analysis, two clusters and one statistically significant cluster was obtained (p value<0.01), having Cal99 and Vacc (Conn and Mass) as its more predominant genotypes and with the highest relative risk.

With respect to the identification of spatial clusters using the multinomial model, results showed two clusters with only the second cluster being statistically significant (p < 0.05). Within this cluster, the Vacc genotype had the highest relative risk (2.55). However, Cal99 was the most predominant in the cluster with respect to the total number (n = 29) of cases. In both analyses broiler farms were the most represented in all the significant and non-significant clusters.

DISCUSSION

Due to its economic and poultry health impact, IB is a well-studied disease in poultry. In contrast, the spatial epidemiology of IB is not well studied especially in contrast to other poultry diseases including Avian Influenza (16). The ability to identify statistically significant spatial, temporal and spatial-temporal clusters of infectious disease is an important tool in public health, disease surveillance and food security (16, 21). With the advent of open-source and non-open source mapping tools and space-time disease surveillance software, timely retrospective analyses of disease outbreaks can be accomplished with little or no cost. Among the commonly used space-time disease surveillance tools which include ClusterSeer, SaTScan, Geosurveillance and the Surveillance package for R, we selected SatScan due to its open source nature, ease of use, well referenced methodologies section and the variety of analyses tools available (14, 15, 24).

In this paper, we explored several different spatial statistical approaches in order to explore practical ways to facilitate identification of clusters of IB. Specifically, based on previous efforts in mapping infectious diseases combined with the high variability of IB genotypes noted in California (10), STPM and multinomial models were identified as appropriate retrospective tools to properly identify spatial-

temporal clustering of IB during the study period (7, 22). These spatial-temporal statistical tools are helpful in identifying both geographical locations and time(s) of year in which IB variants are most likely to occur.

Using the multinomial approach two significant spatial-temporal clusters and one significant spatial cluster was identified. With respect to genotype, results showed that in Cluster 1 on the spatial-temporal analysis and Cluster 2 of the spatial analysis, the vaccine strains had the highest relative risk meaning that more cases of IB associated with the vaccine strain are observed than expected. Consequently, it appears that the vaccine strain has a more significant impact than we would expect with respect to IB. While there was more than one genotype in each cluster it is important to note that significant relative risk were only found for one IB genotype For IB disease surveillance, the above insights regarding the vaccine-like viruses demonstrate the inherent advantage of multinomial models with respect to identification of different genotypes of IB. Specifically, in addition to the geographic and temporal parameters of an IB outbreak, genotypic information can also be provided to help facilitate response efforts.

From a practical perspective, infectious poultry diseases could be analyzed in a retrospective fashion in order to identify clusters of disease and advise the poultry industry about where biosecurity efforts should be focused in the future. In addition, the multinomial clustering models can be used to select appropriate IB vaccine types. Open source spatial analysis tools should be seen as a complementary tool in order to better understand outbreaks with the goal of utilizing the combined data to mitigate outbreaks, focus surveillance efforts and understand spatial-temporal transmission. In the future, at the state and University level, extension of these types of analyses could be incorporated into infectious disease investigations in poultry in order to better inform relevant stakeholders about IB transmission and how best to respond.

REFERENCES

1. Alvarado, I. R., P. Villegas, J. El-Attrache, and T. P. Brown. Evaluation of the protection conferred by commercial vaccines against the California 99 isolate of infectious bronchitis virus. Avian Diseases 47:1298-1304. 2003.

2. Armesto, M., D. Cavanagh, and P. Britton. The Replicase Gene of Avian Coronavirus Infectious Bronchitis Virus Is a Determinant of Pathogenicity. Plos One 4. 2009.

3. Case, J. T., K. W. Sverlow, and B. J. Reynolds. A novel protein polymorphism differentiates the California serotype of infectious bronchitis from other serotypes common to California. Journal of Veterinary Diagnostic Investigation 9:149-155. 1997.

4. Cook, J. K. A., M. Jackwood, and R. C. Jones. The long view: 40 years of infectious bronchitis research. Avian Pathology 41:239-250. 2012.

5. Corsiglia, C. IB Vaccines in California Commercial Poultry In. R. Gallardo, ed. 2017.

6. de Wit, J. J., J. K. A. Cook, and H. van der Heijden. Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathology 40:223-235. 2011.

7. Dong, W., K. Yang, Q. L. Xu, L. Liu, and J. Chen. Spatio-temporal pattern analysis for evaluation of the spread of human infections with avian influenza A(H7N9) virus in China, 2013-2014. BMC Infect. Dis. 17:13. 2017.

8. Dufour-Zavala, D. E. Swayne, J. R. Glisson, J. E. J. E. Pearson, W. M. R. W.M., M. W. Jackwood, and P. R. Woolcock. A laboratory manual for the isolation, identification, and characterization of avian pathogens, 5th ed. In. A. A. o. A. Pathologists, ed., Athens, GA. 2008.

9. Franca, M., P. R. Woolcock, M. Yu, M. W. Jackwood, and H. L. Shivaprasad. Nephritis Associated with Infectious Bronchitis Virus Cal99 Variant in Game Chickens. Avian Diseases 55:422-428. 2011.

10. Gallardo, R. A., O. A. Aleuy, M. Pitesky, G. Senties-Cue, A. Abdelnabi, P. R. Woolcock, R. Hauck, and H. Toro. Variability Assessment of California Infectious Bronchitis Virus Variants. Avian Diseases 60:424-429. 2016.

11. Hauck, R., R. A. Gallardo, P. R. Woolcock, and H. L. Shivaprasad. A Coronavirus Associated with Runting Stunting Syndrome in Broiler Chickens. Avian Diseases 60:528-534. 2016.

12. Jackwood, M. W., D. A. Hilt, C. W. Lee, H. M. Kwon, S. A. Callison, K. M. Moore, H. Moscoso, H. Sellers, and S. Thayer. Data from 11 years of molecular typing infectious bronchitis virus field isolates. Avian Diseases 49:614-618. 2005.

13. Jackwood, M. W., D. A. Hilt, S. M. Williams, P. Woolcock, C. Cardona, and R. O'Connor. Molecular and serologic characterization, pathogenicity, and protection studies with infectious bronchitis virus field isolates from California. Avian Diseases 51:527-533. 2007.

14. Kulldorff, M. SaTScan_Users_Guide. In. 2015.

15. Kulldorff, M., R. Heffernan, J. Hartman, R. Assuncao, and F. Mostashari. A space-time permutation scan statistic for disease outbreak detection. Plos Medicine 2:216-224. 2005.

16. Leo, L., G. Marius, J. M. Wu, C. Christina, H. Muhammad, and X. M. Xiao. Identifying risk factors of highly pathogenic avian influenza (H5N1

subtype) in Indonesia. Prev. Vet. Med. 102:50-58. 2011.

17. Martin, M. P., P. S. Wakenell, P. Woolcock, and B. O'Connor. Evaluation of the effectiveness of two infectious bronchitis virus vaccine programs for preventing disease caused by a California IBV field isolate. Avian Diseases 51:584-589. 2007.

18. McKinley, E. T., M. W. Jackwood, D. A. Hilt, J. C. Kissinger, J. S. Robertson, C. Lemke, and A. H. Paterson. Attenuated live vaccine usage affects accurate measures of virus diversity and mutation rates in avian coronavirus infectious bronchitis virus. Virus Research 158:225-234. 2011.

19. Mondal, S. P., and C. J. Cardona. Genotypic and phenotypic characterization of the California 99 (Cal99) variant of infectious bronchitis virus. Virus Genes 34:327-341. 2007.

20. Moore, K. M., J. D. Bennett, B. S. Seal, and M. W. Jackwood. Sequence comparison of avian infectious bronchitis virus S1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California. Virus Genes 17:63-83. 1998.

21. Muellner, P., J. C. Marshall, S. E. F. Spencer, A. D. Noble, T. Shadbolt, J. M. Collins-Emerson, A. C. Midwinter, P. E. Carter, R. Pirie, D. J. Wilson, D. M. Campbell, M. A. Stevenson, and N. P. French. Utilizing a combination of molecular and spatial tools to assess the effect of a public health intervention. Prev. Vet. Med. 102:242-253. 2011.

22. Park, R., T. F. O'Brien, S. S. Huang, M. A. Baker, D. S. Yokoe, M. Kulldorff, C. Barrett, J. Swift, J. Stelling, and P. Ctr Dis Control. Statistical detection of geographic clusters of resistant Escherichia coli in a regional network with WHONET and SaTScan. Expert Rev. Anti-Infect. Ther. 14:1097-1107. 2016.

23. R.A., G., S. R.C., N. A, S. H.L., and H. B. Pathogenesis and Molecular Biology of an Infectious Bronchitis Virus with Enteric Tropism. Journal of Avian Diseaes 2018 (in review).

24. Robertson, C., and T. A. Nelson. Review of software for space-time disease surveillance. International Journal of Health Geographics 9. 2010.

25. Schikora, B. M., L. M. Shih, and S. K. Hietala. Genetic diversity of avian infectious bronchitis virus California variants isolated between 1988 and 2001 based on the S1 subunit of the spike glycoprotein. Archives of Virology 148:115-136. 2003.

DEVELOPMENT OF A WEB APP BASED PREDICTIVE MODEL UTILIZING LAND SAT IMAGING AND NEXT GENERATION RADAR FOR REAL TIME MONITORING OF WATERFOWL IN CALIFORNIA AS A MECHANISM FOR GUIDING AI SURVEILLANCE

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ABSTRACT

Current surveillance efforts of waterfowl in the US which are the primary reservoir of avian influenza viruses (AIv) are primarily focused on a combination of identification of waterfowl habitat via land use surveys via aerial photography, satellite imagery and various surveillance efforts led by USGS of waterfowl density and species type. Over the last several years our group has developed a multimodel spatiotemporal near real-time approach to track waterfowl and their habitat, including the innovative use of weather radar (NEXRAD) and remotely sensed parameters (Landsat). This remotely sensed land-use data is being used to develop a near real-time predictive mapping model hosted as a web app which integrates multiple layers of remotely sensed data including daily changes in precipitation and temperature in order to develop an interactive spatial model for poultry producers in California. The model allows for the integration of a circular window based on habitat and likely feeding distances in order to help localize the primary location of waterfowl with respect to roosting and feeding locations. The current web app contains two separate maps. One shows the location of historic high density waterfowl habitat using NEXRAD while the other utilizes daily inputs of remotely sensed data integrated into a spatiotemporal model.

As the model is further developed it will integrate all the data into a single risk model. The goal is to allow poultry producers in California and eventually outside of California a real-time interactive mapping web-based tool in order to understand risk with respect to proximity to waterfowl.

INTRODUCTION

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

waterfowl and facilitates a more directed, active approach to AIv surveillance.

Here we present a novel web-app called the California Waterfowl Tracker which incorporates NEXRAD and machine learning using both satellite based environmental data and weather station data including temperature, precipitation, soil moisture content, flooded rice cover, and wetland cover in order to make daily predictions of waterfowl roosting habitat in the central valley of California.

MATERIALS AND METHODS

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

RESULTS AND DISCUSSION

The California Waterfowl Tracker web app is designed to allow producers, backyard enthusiasts, government agencies and other stakeholders a web based tool to better understand the farms spatial relationship to waterfowl which are the primary reservoir of avian influenza. Initial results from the real-time machine learning waterfowl density demonstrate the plasticity of waterfowl habitat based on the predictors described above (Figure 1). These results are important to recognize since current surveillance approaches are fixed and do not take into account daily changes in weather and how those abiotic changes affect waterfowl habitat.

Future directions include the development of a real-time daily monitoring system for the NEXRAD data which will incorporate machine learning models to predict waterfowl distribution beyond NEXRAD range, and real-time processing of NEXRAD and remote sensing data.

REFERENCES

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

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

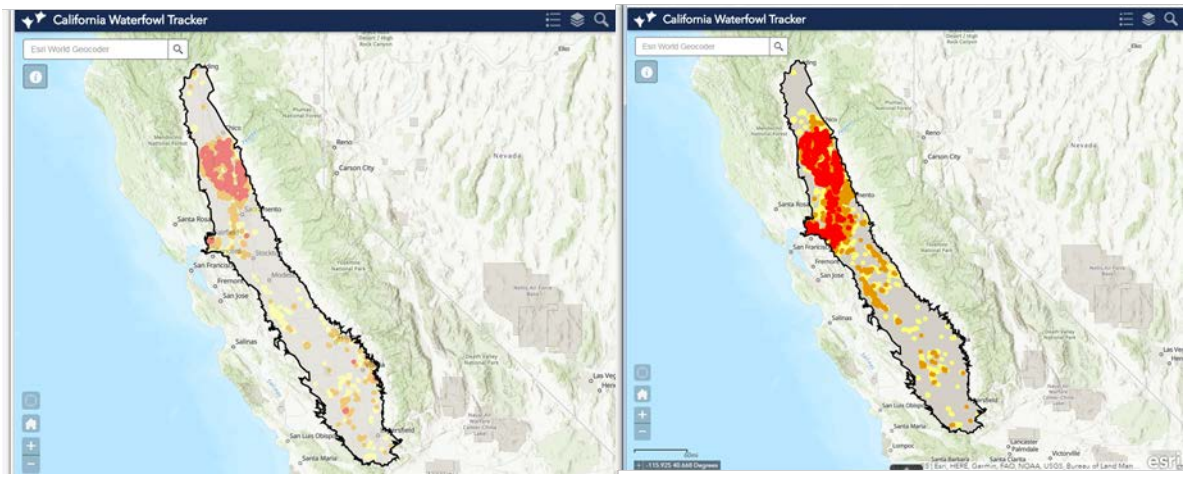
3. Buler, J. J., L. A. Randall, J. P. Fleskes, W. C. Barrow, Jr., T. Bogart, and D. Kluver. Mapping wintering waterfowl distributions using weather surveillance radar. PloS one 7:e41571. 2012.

4. Munster, V. J., and R. A. M. Fouchier. Avian influenza virus: Of virus and bird ecology. Vaccine 27:6340-6344. 2009.

5. Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. Osterhaus, and R. A. Fouchier. Global patterns of influenza a virus in wild birds. Science (New York, N.Y.) 312:384-388. 2006.

6. Peterson, A. T., B. W. Benz, and M. Papes. Highly pathogenic H5N1 avian influenza: entry pathways into North America via bird migration. PloS one 2:e261. 2007.

Table 1. Waterfowl density map of a dry hot day (A) and a rainy cool day (B) in the central valley of California in February and January of 2018 respectively. A map is produced daily and the previous 30 days of maps are stored on the web-app. .



BROAD SPECTRUM PROTECTION AGAINST INCLUSION BODY HEPATITIS USING A BIVALENT LIVE FOWL ADENOVIRUS VACCINE IN BROILER BREEDERS

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ABSTRACT

Inclusion body hepatitis (IBH) in broiler chickens has been reported from many countries. IBH is caused by fowl adenoviruses (FAdVs) among which FAdV2, FAdV7, FAdV8a, FAdV8b and FAdV11 are the most predominant serotypes responsible for outbreaks. Currently, there is no commercially available broiler breeder vaccine for the prevention of IBH in Canada. Therefore, the objective of this study was to develop a live FAdV vaccine combining FAdVs from species E and D. Broiler breeders (n=19) were vaccinated by the oral route at 16 weeks of age with a live bivalent FAdV8a+FAdV11 vaccine at either 10^4 $TCID_{50}$/bird or 10^6 $TCID_{50}$ /bird. Another group (n=12) were kept naïve and comingled with FAdV8b (10^6 $TCID_{50}$/bird) vaccinated breeders. The control group (n=19) received saline. Both doses of FAdV8a+FAdV11 resulted in neutralizing antibodies in breeders by 20 weeks of age, persisting to 50 weeks of age. There was no significant difference of neutralizing antibody levels between the low and high dose groups. All naïve broiler breeders which were comingled with FAdV8b vaccinated broiler breeders seroconverted and produced similar levels of neutralizing antibodies as the FAdV8b vaccinated birds. Cross neutralizing antibodies were produced against FAdV2, FAdV7, FAdV8a, FAdV8b and FAdV11 in broiler breeders which was supported by the survival of their broiler progeny by lethal virus challenge.

In conclusion, we demonstrated the live bivalent FAdV vaccine composed of 10^4 $TCID_{50}$/bird of FAdV8a + FAdV11 is effective in the broad spectrum protection of broilers against IBH caused by group D and E FAdVs. We have also demonstrated that comingling of naïve broiler breeders with vaccinated breeders was an effective immunization technique.

INTRODUCTION

IBH is an acute disease of two to seven week old broiler chickens (5). IBH begins as a sudden onset of mortality which varies between 5-10%, however it is not uncommon to see mortality as high as 30% (2,5,15) with a clinical course of four to five days (5). Depression, ruffled feathers and a crouching position are the prominent clinical signs. IBH is characterized by pale, yellow and hemorrhagic lesions and intranuclear basophilic inclusion bodies in hepatocytes (5). It is caused by multiple serotypes of FAdV (5). FAdVs are categorized into five species (A to E) and are subdivided into 12 serotypes (9). In infected birds, it is possible to isolate two or three serotypes suggesting little cross protection amongst heterologous FAdV species (10). In Canada, outbreaks have been associated with FAdV2, FAdV7, FAdV8 and FAdV11 resulting in mortality up to 30% (6,11). However, commercial vaccines for protection of broiler chickens against more than one serotype are not available in Canada. To protect from multiple FAdV serotypes, multivalent FAdV vaccines need to be evaluated. Therefore, the objectives of our study were to develop an immunization strategy using a combination of live FAdV8a and FAdV11 for broad spectrum protection, determine the effective dose and evaluate seroconversion of naive broiler breeders by comingling with vaccinated birds.

MATERIALS AND METHODS

Viruses used for broiler breeder vaccination (FAdV8a, FAdV8b and FAdV11) were plaque purified and propagated in leghorn male hepatoma cells as previously described (11). Ross broiler breeders were allocated into three groups [group 1 = saline; group 2 = FAdV8a (10^4 $TCID_{50}$ /bird) + FAdV11 (10^4 $TCID_{50}$) /bird; group 3 = FAdV8a (10^6 $TCID_{50}$) + FAdV11 (10^6 $TCID_{50}$)/bird and; group 4 = 50% FAdV8b (10^6 $TCID_{50}$)/bird comingling with 50% non-vaccinated naïve broiler breeders] and were vaccinated orally at 16 weeks of age. Groups 1-3 consisted of 15 females and four males while Group 4 consisted of 20 females and four males. Sera were collected from breeders at 20, 35 and 50 weeks of age from groups 1 to 3 and at 23 weeks from group 4 for virus neutralization testing. Virus neutralization testing was performed to detect neutralizing antibodies against FAdVs as previously described (7). Fertile hatching eggs were collected from broiler breeders between 30-45 weeks of age for broiler progeny challenges. FAdVs from infected liver tissues from field cases of IBH were purified for broiler progeny challenges as described previously (12,14). Broiler progenies (n=30/group) from each broiler breeder group were inoculated intramuscularly in the thigh with 10^7 $TCID_{50}$ of either FAdV2, FAdV7, FAdV8a and FAdV8b at 14 days post-hatch. Birds were kept for 10 d post-challenge. All animal experiments were approved by the University Committee on Animal Care and Supply, Animal Research Ethics Board, University of Saskatchewan following the guidelines of the Canadian Council on Animal Care.

Survival and neutralizing antibody data were analysed using Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) with a significance level of $P<0.05$.

RESULTS

There were no neutralizing antibodies against any of the FAdV serotypes in the saline broiler breeder groups at 20, 35 or 50 weeks of age. Mean neutralizing antibodies against FAdV8a and FAdV11 in both the low and high dose FAdV8a+FAdV11 vaccinated broiler breeder groups were comparable at all time points tested. Naïve broiler breeders in-contact with broiler breeders vaccinated with FAdV8b had comparable neutralizing antibodies at 23 weeks of age. Mean neutralizing antibodies from broilers at 0 d of age from the low and high dose FAdV8a+FAdV11 vaccinated groups were also comparable. Species-specific cross neutralization was detected between FAdV7, FAdV8a and FAdV8b as well as between FAdV2 and FAdV11.

Broiler progeny from all groups were randomly allocated into groups (n=30) and challenged with either FAdV2, FAdV7, FAdV8a, FAdV8b or FAdV11 (10^7 $TCID_{50}$/bird) intramuscularly in the thigh at 14 d of age. Mortality due to IBH peaked at 3-4 d post-challenge in the saline control group. There was significant clinical signs and mortality in the saline group compared to the low or high dose FAdV8a+FAdV11 vaccinated groups. There was no statistically different protection between the low and high dose FAdV8a+FAdV11 vaccinated groups. Additionally, there was no statistically different protection between progeny from the in-contact broiler breeders and FAdV8b vaccinated broiler breeders.

DISCUSSION

IBH is highly prevalent in Canada primarily due to FAdV8a, FAdV8b and FAdV11 (4,11,13). There is currently no commercial IBH vaccine available in Canada. In our study, we used a combination of FAdV8a and FAdV11 broiler breeder vaccine for the protection of broiler progeny against IBH by passive transfer of maternal antibodies. We also evaluated an immunization strategy by comingling naïve broiler breeders with broiler breeders vaccinated with live FAdV8b. After vaccination at 16 weeks of age, we observed antibody responses for the duration of the broiler breeder's life. Neutralizing antibodies were produced against the FAdVs used for vaccination and heterologous FAdVs. The neutralizing antibodies observed in the study were protective as previously reported (8). Neutralizing antibodies in both the low and high dose vaccinated groups were comparable and conferred adequate maternal antibody transfer as evident by progeny survival following virus challenge. Our results are also established by Alvarado *et al*. (1) who demonstrated maternal antibody mediated protection by using a bivalent autogenous FAdV vaccine. We were able to determine that a bivalent FAdV8a+FAdV11 live broiler breeder vaccine at either a low or high dose significantly protected against IBH. Thus, a low dose of FAdV8a+FAdV11 is adequate for an optimal level of antibody response. Additionally, naïve broiler breeders in contact with FAdV8b vaccinated broiler breeders had adequate levels of neutralizing antibodies, due to viral shedding of vaccinated broiler breeders (8). Once neutralizing antibodies are produced, fecal shedding ceases (3,8). Since we vaccinated with a live bivalent vaccine prior to the onset of egg production, there was no vertical transmission to broiler progeny. Thus, a low dose bivalent live FAdV8a+FAdV11 broiler breeder vaccine is achievable to control IBH in broilers in Canada.

(A full-length manuscript has been submitted to *Research in Veterinary Science*.)

REFERENCES

1. Alvarado, I. R., P. Villegas, J. El-Attrache, E. Jensen, G. Rosales, F. Perozo, and L. B. Purvis. Genetic Characterization, Pathogenicity, and Protection Studies with an Avian Adenovirus Isolate Associated with Inclusion Body Hepatitis. Avian Dis. 51: 27–32. 2007.

2. Choi, K., S. Kye, J. Kim, W. Jeon, E. Lee, K. Park, and H. Sung. Epidemiological investigation of outbreaks of fowl adenovirus infection in commercial chickens in Korea. Poult. Sci. 91: 2502–2506. 2012.

3. Clemmer, D. Age-associated changes in fecal excretion patterns of strain 93 chick embryo lethal orphan virus in chicks. Infect. Immun. 5: 60–64. 1972.

4. Dar, A., S. Gomis, I. Shirley, G. Mutwiri, R. Brownlie, A. Potter, V. Gerdts, and S. K. Tikoo. Pathotypic and Molecular Characterization of a Fowl Adenovirus Associated with Inclusion Body Hepatitis in Saskatchewan Chickens. Avian Dis. 56: 73–81. 2012.

5. Fitzgerald, S. D., and M. Hess. Adenovirus Infections. In: Diseases of Poultry, 13th ed. Wiley-Blackwell, Ames, IA. pp. 289–300.

6. Gomis, S., Robert Goodhope, Davor Ojkic, and P. Willson. Inclusion Body Hepatitis as a Primary Disease in Broilers in Saskatchewan, Canada. Avian Dis. 50: 550–555. 2006.

7. Günes, A., A. Marek, B. Grafl, E. Berger, and M. Hess. Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). J. Virol. Methods 183: 147–153. 2012.

8. Gupta, A., S. Popowich, D. Ojkic, S. Kurukulasuriya, B. Chow-Lockerbie, T. Gunawardana, K. Goonewardene, R. Karunarathna, L. E. Ayalew, and K. A. Ahmed. Inactivated and live bivalent fowl adenovirus (FAdV8b+ FAdV11) breeder vaccines provide broad-spectrum protection in chicks against inclusion body hepatitis (IBH). Vaccine. 2017.

9. Harrach, B., M. Benko, G. W. Both, M. Brown, A. Davidson, M. Echavarria, M. Hess, M. Jones, A. Kajon, H. Lehmkul, V. Mautner, S. Mittal, and G. Wadell. Adenoviridae. In: Virus Taxonomy: Nineth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, CA.

10. McFerran, J., and J. Smyth. Avian adenoviruses. Rev. Sci. Tech.-Off. Int. Epizoot. 19: 589–598. 2000.

11. Ojkic, D., E. Martin, J. Swinton, J.-P. Vaillancourt, M. Boulianne, and S. Gomis. Genotyping of Canadian isolates of fowl adenoviruses. Avian Pathol. 37: 95–100. 2008.

12. Ojkic, D., and É. Nagy. The Long Repeat Region Is Dispensable for Fowl Adenovirus Replication in Vitro. Virology 283: 197–206. 2001.

13. Philippe, C., H. Grgiæ, D. Ojkiæ, and É. Nagy. Serologic monitoring of a broiler breeder flock previously affected by inclusion body hepatitis and testing of the progeny for vertical transmission of fowl adenoviruses. Can. J. Vet. Res. 71: 98–102. 2007.

14. Schat, K., and H. Purchase. Cell culture methods. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. Kennett Square: University of Pennsylvania. pp. 223–234.

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

BREAKING DOWN YEAST: YEAST CELL WALL AND MANNANOLIGOSACCHARIDES IN ANIMAL HEALTH

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SUMMARY

Yeast and yeast components have been used in food, brewing, animal nutrition and nutrition supplements for many years. Yeast are diverse and at least 1,500 species of yeast are known but few have been described (1) and can range between pathogenic or safe for human and animal consumption. While there are many species of yeast, there are also many strains within each species (1) so each strain is relatively unique with respect to certain chemical structures (2) that can also change depending on the growth conditions (1). Nevertheless, there is a commonality in the typical structure of yeast to include the inner and outer yeast cell wall (YCW) as well as the internal contents. The YCW is made up of mostly mannoprotein and glucans with a minor amount of chitin (3). Specifically, the outer YCW is made up of mannoproteins that contain mannose (4) which aid in flocculence (5) whereas the inner YCW is made up of different glucans and a small amount of chitin that form the linking scaffold of the yeast wall (4). The internal contents of the yeast, yeast extract, contains many components such as digestible proteins and nucleotides. The focus of this review will be the outer YCW and the function of the mannanoligosaccharides.

The outer YCW primarily consists of mannoprotein side chains that are O- and N-glycosylated (4). These mannoproteins (glycoproteins) contain 15-90% mannose by weight (3) and *Saccharomyces cerevisiae* has four kinds of side chain mannoproteins that differ by structure (3, 5). In all yeasts the mannose-mannose bonds that are found in the mannoproteins are mostly in the alpha configuration and some, such as with the yeast *Pichia guillermondii* (previously known as *Candida guilliermondii*), are in the beta configuration (6). The N-linked mannan are often anchored to a protein on the cell wall with asparagine residues whereas the O-linked mannan are often anchored with serine and threonine (7). These yeast mannan structures can be characteristic for the yeast organism and, as a result, can be fingerprinted (2). The role of these side chains are to influence the ability of the yeast to agglutinate and to elicit antigenic as well as immunogenic responses (5, 7). These mannoprotein structures can be somewhat plastic in nature in that not only do they differ between yeast species and strains but they can also differ depending on their growth conditions (e.g. pH, temperature, time of harvest) (7). However, the role and function of the outer YCW is different than that of the inner YCW, the extract or even the fermented product that the yeast can be grown on.

Bacteria can invade the host through a variety of means that include toxin production to degrade the barrier of the cell lining as well as attachment to the cell lining prior to penetration and invasion. For those bacteria that attach to the cell lining (e.g. *Salmonella*, *Escherichia coli*) they must rapidly and effectively bind the host cell to release virulence factors and avoid removal from the host (8). The adhesion of these bacteria to a host cell has three phases:

1) initial weak adhesion mediated by reversible pairing methods like charge
2) secondary weak adhesion that is transient and give bacteria freedom of movement
3) strong, high-affinity adhesion mediated by receptor and ligand binding with the host cell (8).

The phases of binding occur quickly but the strongest ligand phase occur via fimbriae that bind to sugars on the host cell lining (8-10). There are different fimbrial adhesions that bind to different sugars and different bacteria can have different fimbrial adhesions (9). A widely known adhesion is type 1 fimbria binding mannanoligosaccharides (11). In the 1950s researchers found that certain sugars could inhibit certain bacterial agglutination (12) and in the 1970s a study demonstrated the anti-adhesive effect of mannoside based host receptor analogs with certain bacteria in a murine urinary tract infection model (8). The chemical form and type of mannose sugar can impact the strength of binding with the bacterial fimbriae (9, 11). Some researchers found the ratio of the different mannose side chains on yeast where not correlated with agglutination but rather the chemical form of these side chains (5). Whereas other researchers found some correlation with increase sugar concentration with percentage of agglutination inhibition but that some alpha mannan sugars were significantly more effective at inhibiting bacterial adherence to intestinal epithelial cells *in vitro* (11). In commercial use, pure mannose as a sugar can be fermented by a wide range of intestinal bacteria and is cost prohibitive (9). The outer YCW that contains mannose based sugars in high concentrations (9) and

is presented in a complex manner for better binding (8), is resistant to degradation by intestinal enzymes and does not support bacterial fermentation (9). Consequently, the species and strain of yeast define the cell structure and the structure of the outer YCW define the function and ability to bind to bacterial fimbriae.

Yeast can be used in many ways from live yeast, yeast culture to yeast fermentation products (1, 13). To isolate the YCW, on an industrial scale, the yeast must be grown, lysed and then the non-yeast cell wall components and contaminants must be removed (1). From this isolation the outer YCW can be derived and concentrated to be used for specific roles such as binding type 1 fimbriae bacteria like *E. coli* and *Salmonella* (14). A whole yeast or yeast culture contains the structural components of the yeast but these components may not be presented in the same manner as an isolated cell structure. Many reports, both scientific and practical, have demonstrated the benefits of yeast mannanoligosaccharides (MOS) on the immune system and performance of poultry (10). Further research has led to the development of a "second generation" mannose-rich fraction (MRF) product that has demonstrated enhanced immune modulation and support of gastrointestinal health (10). Meta- and holo-analyses with chicken and turkeys suggest no significant difference in performance when an outer YCW technology was used compared to a preventative antibiotic in the diet and there was significantly better performance compared to no additive in the diet (15-18). Immune modulation with outer YCW MOS or MRF have been studied through the measurement of immune parameters such as antibody levels (19), cytokines (20-22), and immune cells (23-24). Additionally, the action of the outer YCW MOS and MRF technology have been observed to have an impact, even if indirect, on bacterial communities and colonization within the intestinal tract (19, 25-28). The indirect impact may shift microbial populations, communities and diversity within the intestinal tract (19, 26-28). The change in bacterial populations, such as in the cecum, has the potential to alter the structure's functional capability as suggested through predictive functional potential testing of bacterial pathways (28).

Use of yeast and yeast components have long been used in animal nutrition and nutritional supplements of animal health programs. Through better understanding of yeast, specifically *Saccaromyces cerevisiae*, and its structures the yeast can be broken down and different structures isolated. These isolated structures, such as the outer YCW MOS and MRF, have been used to effectively focus on certain challenges within poultry production and optimize intestinal health.

REFERENCES

1. Moran, C., S. Kwiatkowski, A. Yiannikouris and U. A. Thielen. Yeast cell wall components and detection thereof. Patent: *US20110281283A1*. Kentucky, USA, Alltech Inc. 2011.

2. Kocourek, J. and C. E. Ballou. Method for fingerprinting yeast cell wall mannans. J Bacteriol. 100: 1175-1181. 1969.

3. Cohen, R. E. and C. E. Ballou. Mannoproteins: Structure. In: Plant Carbohydrates II, Encyclopedia of Plant Physiology (New Series). W. Tanner and F. A. Loews, eds. Springer, Berlin. pp. 441-458. 1981.

4. Lipke, P. N. and R. Ovalle. Cell wall architecture in yeast: New structure and new challenges. J Bacteriol. 180:3735-3740. 1998.

5. Lyons, T. P. and J. S. Hough. Further evidence for the cross-bridging hypothesis for flocculation of brewer's yeast. J. Inst. Brew. 77: 300-305. 1971.

6. Shibata, N., R. Akagi, T. Hosoya, K. Kawahara, A. Suzuki, K. Ikuta, H. Kobayashi, K. Hisamichi, Y. Okawa and S. Suzuki. Existence of novel branched side chains containing β-1,2 and α-1,6 linkages corresponding to antigenic factor 9 in the mannan of *Candida gulliermondii*. J. Biol. Chem. 271: 9259-9266.; 1996.

7. Katz, R. and C. Brown. Method of palliating lower urinary tract infections by treatment with mannan oligosaccharides. Patent: *US20070244069A1*. California, USA, R. Katz. 2007.

8. Krachler, A. M. and K. Orth. Targeting the bacteria-host interfaces: Strategies in anti-adhesion therapy. Virulence. 4: 284-294. 2013.

9. Spring, P. Effects of mannanoligosaccharide on different cecal parameters and on cecal concentrations of enteric pathogens in poultry. Ph.D. Thesis. Swiss Federal Institute of Technology Zurich, Zurich, Switzerland. 1996.

10. Spring, P., C. Wenk., A. Connolly and A. Kiers. A review of 733 published trials on Bio-Mos®, a mannan oligosaccharide, and Actigen®, a second generation mannose rich fraction, on farm and companion animals. J. Appl. Animal Nutr. 3: e1-8. 2015.

11. Firon, N., S. Ashkenazi, D. Mirelman, I. Ofek and N. Sharon. Aromatic alpha-glycosides of mannose are powerful inhibitors of the adherence of type 1 fimbriated *Escherichia coli* to yeast and intestinal epithelial cells. Infection Immunity. 55: 472-476. 1987.

12. Dugid, J. P., I. W. Smith, G. Dempster and P. N. Edmunds. Non-flagellar filamentous appendages ("fimbriae") and haemagglutinating activity in *Bacterium coli*. J. Path. Bact. 70: 335-348. 1955.

13. Roto, S. M., P. M. Rubinelli and S. C. Ricke. An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. Front. Vet. Sci. 2: 1-18. 2015.

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

15. Hooge, D. M. Turkey pen trials with dietary mannan oligosaccharide: meta-analysis, 1993-2003. Int. J. Poult. Sci. 3: 179-188. 2004.

16. Rosen, G. D. Holo-analysis of the effects of Bio-Mos® in turkey nutrition. Brit. Poult. Sci. 48: 27-32. 2007.

17. Rosen, G. D. Holo-analysis of the effects of Bio-Mos® in broiler nutrition. Brit. Poult. Sci. 48: 21-26. 2007.

18. Hooge, D. M. A. Kiers and A. Connolly. Meta-analysis summary of broiler chicken trials with dietary Actigen™ (2009-2012). Int. J. Poult. Sci. 12: 01-08. 2013.

19. Dawson, K. A. The application of yeast and yeast derivatives in the poultry industry. In: Proc. Australian Poultry Science Symposium, New South Wales, Australia. p. 100-105. 2001.

20. Pescatore, A. J., L. R. Good, M. D. van Benschoten, A. H. Cantor, T. Ao, M. J. Ford, R. S. Samuel, L. M. Macalintal, and K. M. Brennan. Effects of dietary supplementation of Actigen® and threonine on growth performance and intestinal morphology of broiler chicks. In: Proc. XIV European Poultry Conference, Stavanger, Norway. p. 562. 2014.

21. Xue, G. D., S. B. Wu and R. A. Swick. Immune modulation role of yeast cell wall derivative in broilers under subclinical necrotic enteritis challenge. In: Recent Advances in Animal Nutrition–Australia, New South Wales, Australia. 2015.

22. Yitbarek, A., H. Echeverry, J. Brady, J. Hernandez-Doria, G. Camelo-Jaimes, S. Sharif, W. Guenter, J. D. House and J. C. Rodriguez-Lecompte. Innate immune response to yeast-derived carbohydrates in broiler chickens fed organic diets and challenged with *Clostridum perfringens*. Poult. Sci. 91: 1105-1112. 2012.

23. Lourenço, M. C., L. N. Kuritza, R. M. Hayashi, L. B. Miglino, J. F. Durau, L. Pickler and E. Santin. Effect of a mannanoligosaccharide-supplemented diet on intestinal mucosa T lymphocyte populations in chickens challenged with *Salmonella* Enteritidis. J. Appl. Poult. Res. 24: 15-22. 2015.

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

25. M'Sadeq, S. A., S. B. Wu, M. Choct, R. Forder and R. A. Swick. Use of yeast cell wall extract as a tool to reduce the impact of necrotic enteritis in broilers. Poult. Sci. 94: 898-905. 2015.

26. Corrigan, A., K. Horgan, N. Clipson and R. A. Murphy. Effect of dietary supplementation with a Saccharomyces cerevisiae mannan oligosaccharide on the bacterial community structure of broiler cecal contents. Appl. Environ. Microbiol. 77: 6653-6662. 2011.

27. Corrigan, A., K. Horgan, N. Clipson and R. A. Murphy. Effect of dietary prebiotic (mannan oligosaccharide) supplementation on the caecal bacterial community structure of turkeys. Microb. Ecol. 64: 826-836.

28. Corrigan, A., M. de Leeuw, S. Penaud-Frézet, D. Dimova and R. A. Murphy. Phylogenetic and functional alterations in bacterial community compositions in broiler ceca as a result of mannan oligosaccharide supplementation. Appl. Environ. Micrbiol. 81: 3460-3470. 2015.

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THE IMPACT OF NUTRITION IN IMMUNITY AND DISEASE

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ABSTRACT

Reducing the use of antimicrobials in the rearing of poultry can be achieved by enhancing the natural immunity of chickens, thus minimizing disease and pathogen shedding. Intervention of the immune system of the chick in ovo has a dramatic effect on the immunity of the bird post-hatch, increasing its ability to resist a disease challenge. However, it is not only the chick's immune system that must be strengthened but also that of the adult bird. The nutritional status of the laying hen directly influences the nutrient composition of the egg and, in turn, the development and nutritional status of the embryo and hatchling. This has implications for epigenetic regulation of gene expression allowing dividing cells to memorize, or imprint, signalling events that occurred earlier in their development. Folic acid (FA), also known as folate, plays a critical role in nucleic acid and protein synthesis; a deficiency of folate significantly alters the immune response. The body utilizes FA as a methyl group donor that can be incorporated into the DNA and potentially affects gene expression. Toll like receptors (TLR) and the B cell receptor (BCR) recognize antigens, and the major histocompatibility complex (MHC) is used to present the antigen to T cells to initiate an adaptive immune response. Taken together our preliminary results, it is possible to infer that FA has an immunomodulatory effect on chicken B cells, possibly affecting their ability to both recognize antigen through the TLR and BCR pathways and their ability to present antigen via the MHCII presentation pathway.

NUTRITIONAL INTERVENTION

There is a clear link between nutrition and immune capabilities. It is easiest to see when discussing malnutrition and the damage it causes to the organism in general and the immune system specifically. However, the positive link between specific macronutrients and immune capabilities is a strong one. Food components that interact with the immune response have considerable potential to reduce susceptibility to infectious diseases. Examples in poultry diet include amino acids such as arginine or threonine (Kidd MT *et al*., 2001), β-glucans (Lowry, VK, 2005) and zinc and selenium as minerals with a wide spectrum of effects on the immune system (Stahl, J. L, 1989). The race to improve the immune capabilities of production animal such as the chicken through nutritional intervention is crucial, especially since a growing number of countries ban the use of antibiotic growth promoters, as these might have adverse effects on the animals gut microbiota, may hinder nutrient absorption and could contribute to microbial resistance to antibiotics. Another reason that the chicken industry may benefit from immunolgical nutritional intervention is that the selection for rapid growth and egg production dampens the initial immune response (Leshchinsky TV and Klasing KC, 2001a). Nutritional intervention methods such as: 1. Probiotics - the use of bacterial cultures that aid the organism in several ways such as organic acid production (Cherrington *et al*., 1991) and competition with pathogens for nutrient absorption and colonization space. The use of these beneficial bacteria has been shown to have a direct effect on the immune capabilities of the organism. The use of *Lactobacillus fermentum* and *Saccharomyces cerevisiae*, for example, has been shown to effect toll like receptor expression and the T cell population in the chicken intestine (Bai SP *et al*., 2013), and *Clostridium butyricum* has an effect on cytokine production (Zhang L *et al*., 2016). Other than the advantages described above, some commensal bacteria can produce nutrients that are utilized by the organism such as short chain fatty acids (Hamer *et al* 2008) and vitamins such as vitamin K, B12 and B9 also known as folic acid. 2. The second method is to use prebiotics - materials that either benefits the commensal bacteria population of the gut or the host directly. Yeast derived macromolecules (Yitbarek A. *et al*., 2013) and carbohydrates (Munyaka PM *et al*., 2012) have been shown to have an effect on cytokine production and TLR4 expression. The combined administration of probiotics and prebiotics is called synbiotics, and there has been extensive research done on the subject in humans (Chang YS *et al*., 2016), mice (Simeoli R *et al*., 2015) and poultry (Rodríguez-Lecompte JC *et al*., 2012; Madej JP and Bednarczyk M, 2016).

FOLIC ACID IN NUTRITIONAL INTERVENTION

As mentioned above, Folic acid was researched thoroughly as a nutritional immune modulator. This has several reasons. First, it is a substance that can be obtained both nutritionally as well as a metabolite of commensal bacteria such as *Bifidobacterium adolescentis* (Strozzi GP and Mogna L, 2008). Secondly, it is part of several integral pathways including DNA synthesis and methylation, a role, which will be elaborated further. It was found to be extremely important in human nutrition, especially in expecting mothers, where folic acid deficiency may cause neural tube defects (Grosse SD and Collins JS, 2007) as well as problems in producing normal erythrocytes (Carmel R, 2008) and leukocytes (Kaplan SS and Basford RE, 1975). Folic acid supplementation is considered so beneficial, that Canadian and US lawmakers mandated the fortification of cereal grain products with it (Tactacan *et al*., 2010). This led to the exploration of egg enrichment with folic acid through the hen's diet (Seyoum E and Selhub J, 1998). Egg enrichment with FA was a success - dietary FA addition lead to increased egg folate concentrations, with an enriched egg containing approximately 10% of the recommended dietary allowance for adults (Food and Nutrition Board, 1998). Unfortunately, trials to further enrich eggs with folic acid hit an upper limit - folate levels reach a maximum plateau, and beyond that point adding folic aicd to the hens' nutrition did not result in raised folic acid concentration in the egg (House JD *et al*., 2002). However, other factors were found to be altered through folic acid addition to poultry diet, including immunological factors such as T cell population, expression of pro-inflammatory cytokines such as IL-1β and antibody production (Munyaka PM *et al*. 2012),

FOLIC ACID RECEPTORS

There are two major folate transporters. The proton coupled folate carrier (PCFT) mediates intestinal absorption of folic acid. As such, its activity is optimal at low pH values found in the upper part of the intestinal tract and several tumor types (Qiu *et al*., 2006; Kugel Desmoulin *et al*., 2011a). On the other hand, the reduced folate carrier (RFC) is a neutral pH (Sierra, Brigle, Spinella, & Goldman, 1997) membrane transporter of folic acid. It is ubiquitously expressed throughout the body (Whetstine *et al*., 2002) including in B cells (Baslund B *et al*., 2008) and the bursa of Fabricius (Jing M *et al*., 2009) and is crucial for proper embryonic development (Russell, *et al*., 2001). It is considered to be the major folate transport system in mammals (Matherly *et al*., 2007), and has a much higher affinity for 5-methyltetrahydrofolate, the major form of reduced folic acid in the blood, than to unreduced folic acid by several orders of magnitude (Zhao R *et al*., 2001). Interestingly, both receptors' expression is inversely correlated with the concentration of folic acid they encounter. Under folic acid deficiency conditions both receptor types are over expressed (Thakur S *et al*., 2015), while folic acid over-supplementation resulted in reduced presence of RFC and PCFT on cell membranes (Ashokkumar B *et al*., 2007). This decrease is caused by reduced promoter activity, reduced mRNA levels (Ashokkumar B *et al*., 2007), and sequestration of RFC protein in the endoplasmatic reticulum (Hou Z *et al*., 2014).

EPIGENETICS

Although all somatic cells in an organism carry the same genome, each cell type has specific structures and functions that are directed by that cell type's unique gene expression pattern. This gene expression profile is determined in part by the stimuli that the cell receives throughout its life by either the environment or certain signals in the organism (Teitell M., and Richardson B., 2003).

There are two general kinds of genetic expression control mechanisms. The first one relies on specific sequences in the genome like the promoter, inhibitor and enhancer regions. The effects of these factors are relatively transient. When a cell encounters the proper signal it will up- or down regulate the expression of genes relevant to that signal by binding the proper factors to the regulatory sites of those genes. Once the signal is removed, its effect on gene expression will diminish, usually within one cell cycle (Tammen SA *et al*., 2013).

On the other hand, there are some gene expression controls that are not as transient, and not only are they persistent throughout cell life, but they are hereditary, passing form cell to daughter cell. These control mechanisms rely on the structure of the genomic material rather than its sequence. And they are known as epigenetic gene regulators (Tammen SA *et al*., 2013

SUMMARY

It seems that in the context of the adaptive immune system epigenetic control and specifically DNA methylation play a crucial role in cell selection, differentiation and cytokine expression. It acts as a guard; allowing the correct set of genes to be expressed (or silenced) in a timely manner. This control method ensures not only that the cells are

functional, but also that the immune system reacts only to the pathogen presented with the correct cytokine profile (and thus, indirectly, with the correct immunogenic response). On a more general note, DNA methylation, both in the adaptive and innate immune systems, ensures that specific processes will happen only under the right conditions. Actions cannot take place before they are needed, simply because the genes that are part of that action cannot be accessed. Such a powerful control mechanism keeps the immune system in check, and saves the organism from various problems that could arise from an unregulated system.

CHALLENGE STUDY AGAINST INFECTIOUS BRONCHITIS DMV VARIANT FROM CANADA

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SUMMARY

Infectious bronchitis virus infections, predominantly Delmarva (DMV) variant strain, have been causing a false layer syndrome (with 10-55% production reduction) in layers in Ontario, Canada. To aid the resolution of current DMV variant issues in Canada, we conducted a challenge study against the DMV variant to evaluate protection provided by Ceva IBron or Cevac IBron + Mass combination. Prior to the animal study, isolated virus from diagnostic samples were confirmed by S1 genotyping.

INTRODUCTION

Since 2015, the DMV strain of infectious bronchitis virus (IBV) has been emerging as a predominant strain in Ontario, Canada. According to the 2017 Ontario Q4report, the number of DMV cases has increased from 1.85% (2015) to 24.79% (2016) and 46.67% (2017) (1). IBV DMV strain (DMV/1639/11), first reported in the Delmarva area of the United States in 2011 (2), is characterized with nephropathogenic symptoms, increased mortality and respiratory signs. Unlike DMV/1639/11 isolated in the U.S, DMV strains from Ontario are mainly associated with egg production drops (or false layer syndrome) and respiratory symptoms in layers and breeders. False layer syndrome in mature hens is defined by severe egg production drops (50% or less) in healthy looking hens. When chicks are exposed to a new or virulent strain of IBV which they have no maternal antibodies (Abs), the infection (as early as 1 day of age) can damage epithelial cells of the oviduct and results in abnormal oviduct development at maturity. No licensed vaccine for DMV strain is available in Canada, and in the U.S, Cevac® IBron™ , a GA type vaccine (Ceva Animal Health), is the only licensed product that provides protection against the U.S. DMV/1639/11 strain. Also, internal studies have identified that combination of Cevac IBron with Mass type vaccine can induce a broad protection against other IBVs, including Ark type. In this study we evaluated protection against the Canada DMV strain by Cevac IBron vaccine alone or in combination with Mass.

MATERIALS AND METHODS

Virus. Diagnostic samples (tracheal swabs and ceca tonsil), identified as DMV strain by qRT-PCR assay, were received from Animal Health Laboratory, University of Guelph, ON, Canada. Upon reception, samples were tested with IBV qRT-PCRs (3-5) to confirm absence of IBVs other than DMV strain. Two tracheal swabs were further processed for virus isolation in 9-11 day old specific pathogen-free (SPF) embryos and at 48 hours post inoculation, allantoic fluids were collected and re-confirmed with IBV qRT-PCR (1) and a full S1 sequencing (6). Sample 17-2613-2 (original ID17-246438-0004) was selected to be used in following animal experiment.

Vaccines. Cevac IBron (Bronchitis vaccine, Georgia type, Frozen) and Cevac Bron Mass (Bronchitis vaccine, Mass type, Lyophilized) (Ceva Animal Health, Lenexa, USA) were used in this study.

Animal experiment. One-day-old SPF chicks were divided into six groups. Group names and the number of animals in each group are in Table 1. Birds were vaccinated via ocular-nasal route (one dose/bird) at one day-of-age and kept in separate isolation units. At five days post vaccination, 10 choanal swabs were collected from each vaccinated group (Cevac IBron, and Cevac IBron+Mass) to confirm successful vaccination. At 28 days-of-age, Groups 4 - 6 were challenge with Canada DMV virus (17-2613-2), challenge titer: $10^{4.2}$ EID_{50}/bird) via ocular-nasal route. Five days after challenge, all birds were examined for clinical signs and necropsied. During necropsy, choanal swabs were collected for virus isolation and virus detection by qRT-PCR. The animal experimental procedures were approved by the Ceva Biomune Animal Care and Use Committee.

RESULTS

S1 sequence comparison with U.S. DMV1639 strain. Nucleotide and amino acid sequences of the full S1 of Canada DMV strain from this study were analyzed and compared with DMV strains identified in the U.S, including DMV/1639/11. Canada DMV shared 96% similarity of S1 nucleotide sequences and 95% similarity of S1 amino acid sequences with the

U.S DMV strains (whole genome sequence analysis is currently on going).

Detection of vaccine at five days post vaccination. At five days post vaccination, choanal swabs were collected from 10 birds per each vaccine treatment group (Cevac IBron group and Cevac IBron+Mass group) to confirm the replication of vaccine virus in birds. All samples were positives with CT values between 25~27, therefore confirmed successful vaccination.

Clinical signs observed at five days post challenge with Canada DMV (Figure 1). All birds in this experiment were examined for any clinical signs associated with IBV prior to sample collection. Clinical scores were measured based on severity of respiratory symptoms (wheezing and rales). In group 4 (no vaccine/ challenged), most of the birds showed clinical signs varying from slight to marked rales, while the other groups showed no clinical signs.

Clearance of viral RNA at five days post challenge (Figure 2). Choanal swabs were collected in 2ml Phosphate Buffered Saline (PBS) at necropsy, and RNA extraction and 5UTR IBV qRT-PCR(3) was performed. Compared to Group 4 (no vaccine/challenged), which had a mean CT value of 22.12, both the vaccinated and challenged groups (Group 5 IBron vaccinated/challenged and Group 6 IBron+Mass/challenged) showed a 3 log reduction of viral RNA ($p<0.05$). (One way ANOVA Kruskal – Wallis test, Statgraphics (Statgraphics Technology, Inc.)).

Virus recovery in embryonated eggs. No virus was detected in Group 1 (no vaccine/no challenge) and Group 2 (IBron/no challenge), while all samples in Group 4 (no vaccine/challenged) were all positive for virus recovery. In Group 3, (IBron + Mass combination, no challenge), one of 10 samples were positive for IBV recovery, possibly due to uncleared vaccine. Between vaccinated/challenge groups, Group 6 (IBron+Mass/challenged) showed 87% (2/15 samples positive) reduction of virus recovery, compared to 67% (5/15 samples positive) of Group 5 (IBron/challenged). In addition, the severity and frequency of embryo lesions were significantly reduction in both vaccinated/challenged groups compared to the non-vaccinated/challenged group.

CONCLUSION

Challenge study against Canada DMV strain was performed and the protection provided by Cevac IBron and IBron and Mass combination was evaluated. While naïve birds challenged with Canada DMV strain showed severe respiratory signs, vaccinated and challenged birds did not show any apparent clinical signs. Also both qRT-PCR and virus recovery in embryonated eggs showed significant reduction of viral RNA as well as virus recovery in both vaccinated and challenged birds. Although IBron provided protection against the variant DMV challenge, the IBron and Mass vaccines in combination provided higher protection against Canada DMV challenge than the IBron alone.

REFERENCES

1. Ontario Animal Health Network (OAHN) Poultry Expert Network Quarterly Producer Report. Quarter 4, 2017. August 1st – October 31st 2017.

2. Gelb, J. Jr., Ladman B.S., Pope, C.R., Ruano, J.M., Brannick, E.M., Bautista, D.A., Coughlin, C.M., Preskenis, L.A. Characterization of nephropathogenic infectious bronchitis virus DMV/1639/11 recovered from Delmarva broiler chickens in 2011. Avian Dis. 57(1):65-70, 2013.

3. Callison, S.A., Hilt, D.A., Boynton, T.O., Sample, B.F., Robison, R., Swayne, D.E., Jackwood, M.W. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J.Virol.Methods. 138: 60-65, 2006.

4. Roh, H.J., Hilt, D.A., Williams, S.M., Jackwood, M.W. Evaluation of infectious bronchitis virus Arkansas-type vaccine failure in commercial broilers. Avi. Dis. 57(2):248-259, 2013.

5. Roh, H.J., Jordan, B.J., Hilt, D.A., Jackwood, M.W. Detection of infectious bronchitis virus with the use of real-time quantitative reverse transcriptase-PCR and correlation with virus detection in embryonated eggs. Avian Dis. 58(3):398-403, 2014

6. Lee, C.W., Hilt, D.A., Jackwood, M.W. Redesign of primer and application of the reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism test to the DE072 strain of infectious bronchitis virus. Avian Dis. 44(3):650-654, 2000.

Table 1. Animal treatment groups.

Group	Vaccine	Challenge	# of animal
1	No vaccine	No challenge	10
2	Cevac IBron	No challenge	10
3	Cevac IBron + Mass	No challenge	10
4	No vaccine	Canada DMV (17-2613-2)	15
5	Cevac IBron	Canada DMV (17-2613-2)	15
6	Cevac IBron + Mass	Canada DMV (17-2613-2)	15

Figure 1. Clinical signs scored at five days post challenge.

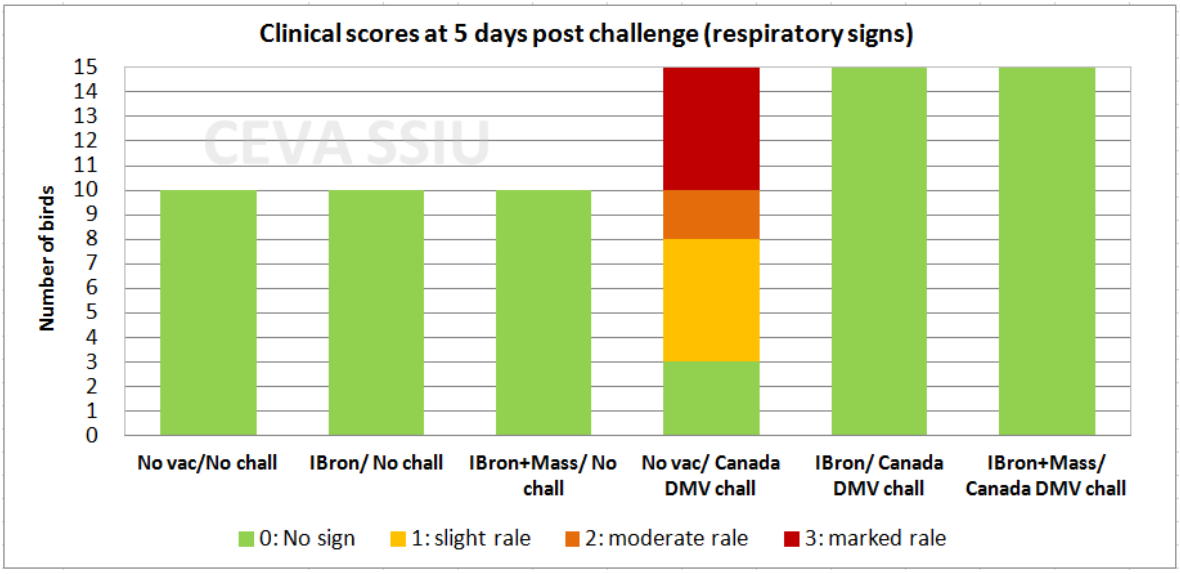
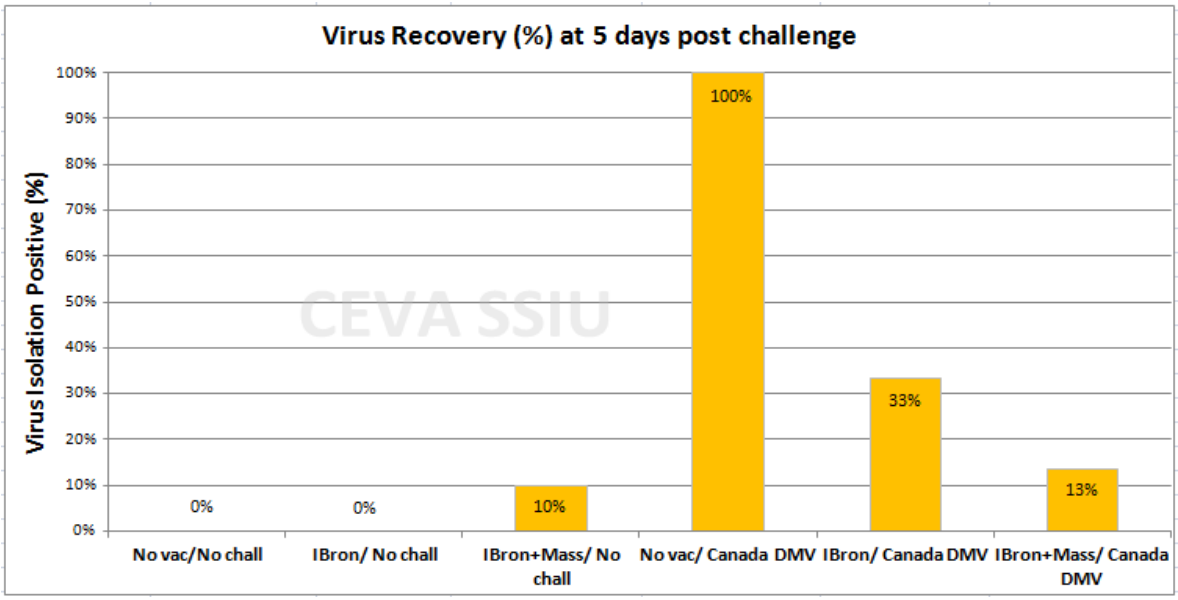


Figure 2. Virus recovery from samples collected at five days post challenge using embryonated eggs.



HISTOMONIASIS IN BROILER BREEDERS: AN EMERGING ISSUE?

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SUMMARY

Histomoniasis has been described for more than 100 years and is an important economic disease problem in turkeys, but less so in layer chickens and broiler breeder pullets. It is a parasitic disease of the ceca and liver caused by *Histomonas meleagridis* and is carried by the cecal worm *Heterakis gallinarum.* Histomoniasis is commonly and erroneously called blackhead disease (1). The FDA's disallowance of the use of the nitroimidazole antihistomonals in the 1990s left no available products for treatment of the disease (2). The more recent voluntary manufacturer discontinuation of the phenylarsonic acids such as roxarsone and nitarsone have made control of this disease even more difficult (3). While most histomoniasis in chickens has historically been in reared pullets, recent reports of significant mortality in broiler breeders during the early laying period have been reported. Several confirmed diagnostic cases of acute histomoniasis in adult broiler breeders in the southeastern broiler belt causing significant mortality and production loss have been reported. The majority of these cases have presented as a rise in mortality over the normal mortality rate of .3% per week along with a mild decrease in egg production. Most have occurred relatively early in the laying cycle and seem to have been initiated by cecal coccidiosis. Necropsy results include whitish cecal cores and the occasional liver lesion, but liver lesions have been sporadic, perhaps indicating minimal pathology from the histomonads and a failure to properly immunize against *Eimeria tenella* in the pullet stage.

DISCUSSION

Recent regulations limiting therapeutic treatment options in food animals have created challenges for veterinarians attempting to establish disease control programs. The loss of the nitromidazole antihistomals have made it difficult to treat histomonad infection in poultry. Also, the voluntary withdrawal of the phenylarsonic acids such as roxarsone and nitarsone have made it impossible to prevent the disease pharmaceutically and compounds the issue of decreasing availability of therapeutics. To further complicate the issue, coccidiosis control continues to become more difficult with limited options for vaccination or chemoprophylaxis in broiler breeder pullets. This perfect storm of therapeutic inadequacy, along with increasing helminthic resistance to available de-wormers, has made it difficult to control histomoniasis and most likely had significant influence on the emergence of the adult blackhead cases being observed in breeders. In 2016, a mini-symposium on histomoniasis was held in conjunction with the annual meeting of the American Association of Avian Pathologist in San Antonio, TX., to discuss the increasing importance of blackhead disease in poultry in turkeys and broiler breeders. Histomoniasis was reported to be a disease of significance economic loss in broiler breeders and in one outbreak, a mortality of more than 6% was noted (3).

CASE REPORTS

In 2017, there was an increase in the number of cases of blackhead seen at the Poultry Diagnostic and Research Center in Athens, GA. Of interest were the cases of an infection in young breeders between 25-30 weeks of age. This was confirmed by reports of similar cases investigated by the author throughout the southeastern broiler belt. The cases were similar in that they occurred as the birds were climbing toward peak production, with a moderate rise in daily mortality (>.3%/week) and delay in reaching peak production standards. Most of the mortality showed cecal cores, and oocysts of *E. tenella* could be found if the case was fresh or early in the pathogenesis. Liver lesions may or may not be present and at times did not fit the pattern of circular surface lesions. Upon collecting history from these farms, it appeared that a late breaking *E. tenella* infection was most likely initiating this event and providing an entrance for the histomonads which were being harbored in the often present *Heterakis* nematodes. Because cecal worms could not be visualized in all affected birds, confirmation of this theory was not possible.

TREATMENT AND CONTROL

Because the lesions typical of cecal coccidiosis were present in so many of the birds examined, there is reason to question the coccidiosis control program of the pullet flocks. One of the main points from the mini-symposium in San Antonio was the importance

of cecal coccidiosis control in pullet flocks. A coccidia vaccine, (primarily Coccivac®-D), is used in the vast majority of pullet rearing programs and when administered and managed properly, should provide protection from coccidiosis challenge when moving birds to the hen house. The observation that so many of the affected hens seem to be showing signs of *E. tenella* after housing suggests that there is a problem with developing adequate immunity. Other non-related cases submitted to PDRC in younger birds have confirmed that management of today's broiler breeder pullet is not ideal. The fact that breeding stock undergoes continuous genetic selection for progeny performance and not necessarily for pullet robustness seems to magnify management errors in pullet house management. Therefore, it is imperative that the pullet coccidia vaccination programs be reviewed along with intensive helminth control and pullet house floor management to prevent this from occurring when the birds are beginning to lay eggs.

REFERENCES

1. Hess, M. and McDougald, L. Histomoniasis (Blackhead) and Other Protozoan Diseases of the Intestinal Tract. In: Diseases of Poultry. 13th Edition. Pp. 1172-1178.

2. McDougald, L. Blackhead Disease (Histomoniasis) in Poultry: A Critical Review. Avian Dis. 49:462-476. 2005.

3. Clark, S. and Kimminau, E. Critical Review: Future Control of Blackhead Disease (Histomoniasis) in Poultry. Avian Dis. 61:281-288. 2017.

ANTIMICROBIAL RESISTANCE AMONG *E. COLI* STRAINS ISOLATED FROM TWO GENETIC LINES OF LAYING HENS

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INTRODUCTION

Mexico is the number one consumer of eggs in the world and ranks number six in production of eggs, being achieved because the flock of layers amounts to 154 million birds, and more than half of them (55%) are located in the State of Jalisco (1). The success of the Mexican table egg industry is due to a combination of factors, such as specialized genetic lines, nutrition, biosecurity programs, infrastructure, and preventive medicine. However, to satisfy the demand for this product, the industry is in constant jeopardy.

Laying hens are exposed to a number of pathogens, such as bacteria, viruses, and parasites. Among bacteria, *Escherichia coli* has become one of the main causes of economic losses and mortality in laying flocks. This is a genetically diverse bacterium, and is widespread in the intestine of animals and humans, comprising non-pathogenic intestinal strains and pathogenic strains responsible for intestinal and extra-intestinal disease (2). Those strains able to cause disease in chickens are known as avian pathogenic *E. coli* (APEC). APEC is mainly associated with extraintestinal infections known as colibacillosis The most common lesions associated with colibacillosis are perihepatitis, airsacculitis, and pericarditis, although other syndromes such as osteomyelitis, arthritis, yolk peritonitis, peritonitis/salpingitis (SPS syndrome), coligranuloma, omphalitis, and cellulitis can also be found (3).

Extraintestinal infections due to *E. coli* are an important cause of morbidity, mortality, and increased health care costs (4). The rising prevalence in *E. coli* resistant to all first-line antibiotics makes treatment of such infections increasingly challenging (5). Antimicrobial resistance is of main concern, since antimicrobial-resistant bacteria can pose a risk to human health because of potential treatment failure, loss of treatment options, and increased likelihood and severity of disease (6).

Traditionally, *E. coli* infections have been treated with antimicrobials; however, recently a high rate of antimicrobial resistance has been reported in field isolates. For this reason, 66 strains of *E. coli* isolated from pullets of two different genetic background were compared using the disk diffusion technique against the main antimicrobial of interest.

MATERIAL AND METHODS

Strains. Sixty-six strains of *E. coli* were isolated from two flocks belonging to different genetic lines located at Tepatitlan, Jalisco. Strains were isolated from pullets during the first two weeks of life. Daily, mortality associated with yolk sac infection were collected and sent to the laboratory to be necropsied. Samples of liver, lung, yolk sac, and bone marrow were incubated at 37°C on nutrient broth during 24 h and subsequently streaked onto MacConkey Agar and incubated under the conditions previously described. Colonies were isolated in pure culture and identified using biochemical tests such as TSI, citrate, LIA, urea, and SIM. Strains identified as *E. coli* were store on Dorset media until they were analyzed. Twenty-six were isolated from genetic line identified as A and 40 were isolated from line B.

Antibiogram was performed according to the technique described by Bauer and Kirby (1966) using nine different antibiotics: doxycycline (D), ceftiofur (CFT), ceftriaxone (CTX), enrofloxacin (ENR), sulfamethoxazole/trimethoprim (STX), amoxicillin/clavulanic acid (ACA), azithromycin (AZT), gentamicin (G), and amikacin (AMK).

RESULTS

Considering all strains, a high level of resistance was found among them (Table 1). The highest level was detected against doxycycline (82%), followed by ceftiofur (77%) and ceftriaxone (73%), on the other hand, the lowest resistance was seen against amikacin (5%) and gentamicin (6%); however, this resistance varied depending on the genetic line, Line B was more resistance compared with line A in all tested antimicrobials as it can be seen in Table 2; in some cases, the difference were more evident, as in enrofloxacin case, where resistance among line A was 31%, compared with 90% in line B.

In order to construct a resistance profile pattern, intermediate strains were considered resistant, this information can be seen in Table 3. Twenty-seven

different patterns were observed, the most resistant group was identified as "A" that only contains one strains from line B, that was resistant to all tested antimicrobial, whereas, the most sensitive strain belongs to profile "Z" that was sensitive to all antimicrobial tested and were isolated from line A. On the other hand, the most common profile was identified as "H" that shows resistance against 6 antimicrobials, and contains 10 stains, 8 from line B and 2 from A.

DISCUSSION

Antimicrobial resistance in bacteria, particularly those isolated from food animals, has increased worldwide. This increase has been attributed to a number of factors, including misuse of antimicrobial drugs in humans and animals (8). In fact, food-producing animals have been documented as a reservoir of resistant bacteria. As it was seen in the present work, a high level of resistant bacteria has been found. Particularly, against doxycycline was considerable high, since 82% of analyzed strains were resistant to this antimicrobial agent. This was not surprising, since it has been reported that tetracycline is a kind of broad-spectrum antibiotic that can play bactericidal activity by preventing bacterial protein synthesis. It has been widely used in the prevention and treatment of poultry diseases. However, the application of the tetracycline antibiotics directly leads to producing drug resistance (9)

On the other hand, over the last decade, a high incidence of extended spectrum cephalosporin (ESC) resistance has been observed in different genera of Enterobacteriaceae and has become a serious public health problem worldwide (10). In the present work a high resistant rate was seen against ceftiofur. This situation could be related to the fact that in Mexico this antimicrobial is not prohibited. Ceftiofur is a third-generation cephalosporin antibiotic used to treat cattle and swine for bacterial infection of the respiratory tract. It is not authorized for use in poultry within the European Union (11).

The rapid increase in the rate of antimicrobial-resistant bacteria (AMR) reinforced is currently one of the most serious public health threats, as recognized by the World Health Organization (12). For this reason it is necessary to generate information about the situation in Mexico in this field, since resistance trends in Gram-negative bacilli are particularly alarming due to limited antibiotic options to treat infections caused by some organisms such as Enterobacteriaceae that are becoming resistant to nearly all available antimicrobials, including carbapenems. For this reason, veterinarians who oversee table-egg farms should be very cautious in the use of this kind of therapeutic agents

REFERENCES

1. Union Nacional de avicultores. http://una.org.mx/english/index.php/overview/la-afeccion-de-la-influenza-aviar-h7n3-en-las-exportaciones-avicolas. 2014.

2. Solà-Ginés M, Cameron-Veas K, Badiola I, Dolz R, Majó N, Dahbi G, Viso S, Mora A, Blanco J, Piedra-Carrasco N, González-López JJ, Migura-Garcia L. Diversity of Multi-Drug Resistant Avian Pathogenic Escherichia coli (APEC) Causing Outbreaks of Colibacillosis in Broilers during 2012 in Spain. LoS One. 2015 Nov 23;10 (11):e0143191.

3. Braga JFV, Chanteloup NK, Trotereau A, Baucheron S, Guabiraba R, Ecco R, Schouler C. Diversity of Escherichia coli strains involved in vertebral osteomyelitis and arthritis in broilers in Brazil.BMC Vet Res. 2016 Jul 14;12(1):140.

4. Russo TA, Johnson JR. Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem.Microbes Infect. 2003 Apr;5(5):449-56.

5. Johnson JR, Porter SB, Johnston B, Thuras P, Clock S, Crupain M, Rangan U. Extraintestinal Pathogenic and Antimicrobial-Resistant Escherichia coli, Including Sequence Type 131 (ST131), from Retail Chicken Breasts in the United States in 2013. Appl Environ Microbiol. 2017 Mar 2;83(6). pii: e02956-16.

6. Jiménez-Belenguer A, Doménech E, Villagrá A, Fenollar A, Ferrús MA. Antimicrobial resistance of Escherichia coli isolated in newly-hatched chickens and effect of amoxicillin treatment during their growth. Avian Pathol. 2016 Aug;45(4):501-7.

7. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 1966;45:493-6.

8. Adenipekun EO, Jackson CR, Oluwadun A, Iwalokun BA, Frye JG, Barrett JB, Hiott LM, Woodley TA. Prevalence and Antimicrobial Resistance in Escherichia coli from Food Animals in Lagos, Nigeria. Microb Drug Resist. 2015 Jun;21(3):358-65.

9. Zhang T, Wang CG, Lv JC, Wang RS, Zhong XH. Survey on tetracycline resistance and antibiotic-resistant genotype of avian Escherichia coli in North China.Poult Sci. 2012 Nov;91(11):2774-7.

10. Shahada F, Chuma T, Kosugi G, Kusumoto M, Iwata T, Akiba M. Distribution of extended-spectrum cephalosporin resistance determinants in Salmonella enterica and Escherichia coli isolated from broilers in southern Japan. Poult Sci. 2013 Jun;92(6):1641-9.

11. Heinrich K, Chan D, Fussell RJ, Kay JF, Sharman M. Can the unauthorised use of ceftiofur be detected in poultry? Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2013;30(10):1733-8.

12. Alonso CA1, Zarazaga M1, Ben Sallem R2, Jouini A3, Ben Slama K2, Torres C. Antibiotic resistance in Escherichia coli in husbandry animals: the African perspective. Lett Appl Microbiol. 2017 May;64(5):318-334.

Table 1. Results of antimicrobial disk diffusion test of 66 *E. coli* strains associated with yolk sac infection isolated from two genetic lines of laying hens.

Antimicrobial	Sensible		Intermediate		Resistant	
	Number (%)					
Doxycycline	3	(5)	9	(14)	54	(82)
Ceftiofur	10	(15)	5	(8)	51	(77)
Ceftriaxone	7	(11)	11	(17)	48	(73)
Enrofloxacin	18	(27)	4	(6)	44	(67)
Sulfamethoxazole/trimethoprim	26	(39)	9	(14)	31	(47)
Amoxicillin/clavulanic acid	17	(26)	22	(33)	27	(41)
Azithromycin	38	(58)	18	(27)	10	(15)
Gentamicin	55	(83)	7	(11)	4	(6)
Amikacin	63	(95)	0	(0)	3	(5)

Table 2. Comparison of antimicrobial resistance among two genetic lines of laying hens of 66 *E. coli* strains associated with yolk sac infection isolated from

	Line A (n=26)		Line B (n=40)	
	Number (%*)			
Doxycycline	20	(77)	34	(85)
Ceftiofur	16	(62)	35	(88)
Ceftriaxone	15	(58)	33	(83)
Enrofloxacin	8	(31)	36	(90)
Sulfamethoxazole/trimethoprim	9	(35)	22	(55)
Amoxicillin/clavulanic acid	10	(38)	17	(43)
Azithromycin	4	(15)	6	(15)
Gentamicin	1	(4)	3	(8)
Amikacin	0	(0)	3	(8)

*Percentage was obtain considering the number of strain isolated from each genetic line as 100%.

Table 3. Antimicrobial profile of 66 *E. coli* strains associated with yolk sac infection isolated from two genetic lines of laying hens.

Profile	Line A	Line B	Amikacin	Gentamicin	Azithromycin	Amoxicillin/clavulanic acid	Sulfamethoxazole/trimethoprim	Enrofloxacin	Ceftriaxone	Ceftiofur	Doxycycline
A		1	R	I	R	R	R	R	R	R	R
B	3	2	S	I	I	I	R	R	R	R	R
C	2	7	S	S	I	R	I	R	I	R	R
D		3	S	I	S	I	R	R	R	R	R
E		2	R	S	R	R	R	R	R	S	R
F		1	S	R	I	I	R	S	R	R	R
G	2	1	S	S	I	I	I	S	R	R	R
H	2	8	S	S	S	I	R	R	R	R	R
I		1	S	S	R	R	S	R	R	R	R
J		1	S	S	R	S	R	R	R	R	R
K	1	1	S	S	S	S	R	I	R	R	R
L	2		S	S	S	R	R	S	I	I	R
M		2	S	S	S	R	S	R	R	R	R
N		1	S	S	I	S	S	R	R	R	R
Ñ		1	S	I	S	S	S	R	R	R	R
O	3	3	S	S	S	S	S	R	R	R	R
P	2		S	S	S	R	S	S	R	R	I
Q	1		S	S	I	R	S	S	R	S	R
R	2		S	S	S	R	S	S	I	I	S
S	1		S	S	I	I	S	S	S	S	R
T	1		S	S	S	I	I	S	R	R	R
U		2	S	S	S	R	S	R	I	R	R
V		1	S	S	I	I	S	R	R	R	R
W	1		S	S	R	S	S	S	S	S	R
X		1	S	S	S	S	S	R	s	S	I
Y	2	1	S	S	S	S	S	S	S	S	R
Z	1		S	S	S	S	S	S	S	S	S

DETECTION OF VIRULENCE GENES AMONG *E. COLI* STRAINS ISOLATED FROM TWO GENETIC LINES OF LAYING HENS

F. Ruiz Jimenez

INTRODUCTION

Colibacillosis is the most common infectious bacterial disease of poultry and collectively, *E. coli* infections in their various forms are responsible for significant economic losses (10). *E. coli* is one of the most important bacteria of the gut microbiota in a wide variety of animals. In chickens, there are about 10^9 colony forming units (CFU) of bacteria per gram of feces and of these, 10^6 CFU are *E. coli*. It has also been commonly isolated from the upper respiratory tract and it´s present on the bird´s skin and feathers (9). Despite *E. coli* is a normal inhabitant of the digestive tract, there are some strains capable of causing infections in other parts of the organism since they can survive outside the intestine. These strains are grouped in a category named extraintestinal pathogenic *E. coli* (ExPEC), which includes the pathotypes uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC). The first two pathotypes affect humans and the latter one affects birds. Despite the host difference, these strains are closely related and share some virulence properties (7).

APEC strains are capable of causing a wide variety of extraintestinal infections affecting all types of birds like broilers, layers and, breeders (10). For a long time, colibacillosis was considered a secondary or opportunistic disease following a primary infection with respiratory pathogens and/or unfavorable environmental conditions, however, there´s increasing evidence that the APEC strains have all the properties to be primary pathogens (14).

Since *E. coli* is a normal inhabitant of the gut in poultry, it´s difficult to know if an isolate belongs to a commensal or a pathogenic strain. This has raised the interest of many investigators that have tried to identify specific characteristics that can differentiate commensal from pathogenic *E. coli* strains, concluding that traits like colicin V (ColV) production, type F1 fimbriae expression, embryo lethality, and belonging to certain serotypes like O1, O2, and O78 are most commonly observed in APEC strains compared to the commensal ones (4,10,12,13). This proves that APEC strains have gained virulence genes that can be grouped in pathogenicity islands (PAI´s) located on chromosomes or plasmids. The presence of these PAI´s is considered a defining characteristic of the APEC strains and has been used as a significant diagnostic method (5,10). Some of the most common genes found in these PAI´s are *iroN, iss, hlyF, iutA, ompT,* and *tsh*, which are located in the ColV plasmid (1,2,5,6).

The objective of this study was to identify six virulence genes closely related to APEC strains (*iroN, iss, hlyF, iutA, ompT,* and *tsh)* in sixty-six strains of *E.coli* isolated from two genetic lines (A and B) of layer hens from a farm located in Jalisco, Mexico. This farm has always used hens from the genetic line B but due to the colibacillosis problems they decided to introduce a new flock of hens from the genetic line A and compare the *E. coli* strains from both lines. The results will allow knowing the virulence mechanisms used by *E. coli* in both genetic lines and compare their pathogenicity. They will also be useful to know which genetic line is more convenient to use in that geographical zone and new preventive strategies could be developed.

MATERIALS AND METHODS

Bacterial strains. Sixty-six strains of *E. coli* conserved on Dorset egg medium were used. These strains were isolated from pullets of two genetic lines; 40 of them belonged to the genetic line A while the 26 remaining were from the genetic line B. The strains were isolated from different organs as the yolk sac, bone marrow, lungs, and liver.

Detection of virulence genes. A PCR test following the protocol of *Lopez Saucedo et al.* (8) was used to identify each of the six virulence genes. The reaction conditions were the following: 1mM Tris-HCl-KCl, 2 mM $MgCl_2$, 0.2 mM dNTP´s, 0.5 U Taq DNA polymerase (Invitrogen), 1.4 µM each primer, 13.9 µL distilled water, and 2 µL of DNA template. The reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems) using the following cycle parameters: 95°C for 13 min; 32 cycles of 94°C for 30 s, 63°C for 30 s, 68°C for 3 min; and a final cycle of 72°C for 10 min.

Detection of virulent strains. According to Johnson *et al.* (5), if we observed a strain with three or more virulence genes it was considered as virulent.

RESULTS

Among the 66 strains studied, 44 strains (66.6%) had three or more virulence genes so they were considered as virulent. Within the virulent strains, 25 were isolated from the genetic line A while the 19 remaining were obtained from the genetic line B. It was interesting that the great majority of this strains, in both genetic lines, showed similar distribution patterns of virulence genes (Table 1). For example, within the virulent strains isolated from the genetic line A, the combination of genes #7 was found in 56%; while the 42% of the virulent strains from the genetic line B, presented the combination of genes #1.

The virulence gene with the highest prevalence in all the strains was *iutA*, which was found in 72% of them (n=48), while the lowest prevalence gene was *tsh* with just 15.2% (n=10). Among the virulent strains, *hlyF* had a prevalence of 100% (n=44), and in all of them, except for two, it was associated with the genes *iutA* and *ompT*. In addition, the genes *iutA* and *ompT* were only found in virulent strains; as well as *tsh*, that despite being the lowest prevalence gene, it was only present in virulent strains and nine out of the ten strains where it was found had the whole six virulence genes. The complete prevalence of the genes in the virulent strains is shown in the Table 1.

The most common combination of genes in both the total and virulent strains was the one that involved the gene *hlyF* with *iutA* and *ompT*. This combination was found in 42 of the total strains (63.6%), and all of them were virulent. Also, only this association with no other gene present was found in 19 out of the 42 strains (45.2%), of which 14 were isolated from the genetic line A, mainly from the yolk sac and bone marrow.

Regarding the organ from which the strains were obtained, we observed a great predominance of the isolations from the yolk sac and bone marrow among the strains of the genetic line A, constituting the 80% of the total between both organs. On the other hand, the organs from which the strains of the genetic line B were isolated are more diverse and none of them by itself represents more than the 45% of the total. Nevertheless, it´s interesting to mention that only one strain was isolated from the bone marrow, while a large part of them was obtained from the lungs. This represents a great contrast between the strains of both genetic lines.

DISCUSSION

The prevalence of the genes identified in this study differs significantly from other investigations and publications like the ones from previous studies. (5,11). This could be a suggestion that the genotype and the virulence mechanisms of the APEC strains isolated in Mexico are different than the ones presented in the strains studied by other authors. Nevertheless, according to a previous study (15), this could be due to a variety of reasons like the geographical zone, the type and age of the birds, and the form of the infection that they are presenting. Therefore it´s necessary to perform more investigations about APEC strains in Mexico, obtained from different locations and different types of birds; also trying to identify a greater amount of virulence genes. This will allow having more information about the APEC strains in Mexico and trying to clarify the virulence mechanisms used by them.

It´s interesting to note the high frequency of strains where some specific combinations of genes were found. Indeed, more than 88% of the virulent strains of each genetic line can be grouped in only three patterns of genes, as well as in a great part of them the originating organs are the same. This could mean that there are certain groups of pathogenic *E. coli* that are predominant in each genetic line and could be a key point to develop a vaccine able to protect the birds according to their bloodline. In accordance with Ghunaim et al. (3), different kinds of vaccines could be developed; like inactivated and subunit vaccines. Both have shown positive results, but the subunit vaccines confer homologous and heterologous immunity in contrast with the inactivated vaccines that only confer homologous protection. Serotyping of the strains would be really useful in order to confirm if the strains with shared patterns of genes are from the same serotype and being able to develop a vaccine aimed at that predominant types. This would confer the birds an effective protection against the disease in this specific geographic region and could prevent great economic losses to the Mexican poultry industry

REFERENCES

1. Dissanayake, D. R. A., S. Octavia, and R. Lan.Population structure and virulence content of avian pathogenic Escherichia coli isolated from outbreaks in sri lanka.Vet. Microbiol. 168: 403–412.2014.

2. Fairbrother, J. M., C. M. Dozois, M. Dho-moulin, A. Bre, C. Desautels, and R. O. Y. C. Iii.Relationship between the Tsh Autotransporter and Pathogenicity of Avian Escherichia coli and Localization and Analysis of the tsh Genetic Region.Infect. Immun. 68: 4145–4154.2000.

3. Ghunaim, H., M. A. Abu-Madi, and S. Kariyawasam.Advances in vaccination against avian pathogenic Escherichia coli respiratory disease: Potentials and limitations.Vet. Microbiol. 172.2014.

4. Gibbs, P. S., J. J. Maurer, L. K. Nolan, and R. E. Wooley.Prediction of chicken embryo lethality with

the avian Escherichia coli traits complement resistance, colicin V production, and presence of the increased serum survival gene cluster (iss).Avian Dis. 47: 370–9.2003.

5. Johnson, T. J., Y. Wannemuehler, C. Doetkott, S. J. Johnson, S. C. Rosenberger, and L. K. Nolan.Identification of minimal predictors of avian pathogenic Escherichia coli virulence for use as a rapid diagnostic tool.J. Clin. Microbiol. 46: 3987–3996.2008.

6. Johnson, T. J., Y. M. Wannemuehler, and L. K. Nolan.Evolution of the iss gene in Escherichia coli.Appl. Environ. Microbiol. 74: 2360–2369.2008.

7. Kaper, J. B., J. P. Nataro, and H. L. T. Mobley.Pathogenic Escherichia coli.Nat. Rev. Microbiol. 2: 123–140.2004.

8. Lopez-Saucedo, C., J. F. Cerna, N. Villegas-Sepulveda, R. Thompson, F. R. Velazquez, J. Torres, P. I. Tarr, and T. Estrada-Garcia.Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic Escherichia coli.Emerg. Infect. Dis. 9: 127–131.2003.

9. Lutful Kabir, S. M.Avian colibacillosis and salmonellosis: A closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns.Int. J. Environ. Res. Public Health 7: 89–114.2010.

10. Nolan, L. K., J. H. Barnes, J.-P. Vaillancourt, T. Abdul-Aziz, and C. M. Logue.Colibacillosis. In: Diseases of Poultry, 13th Editi. pp. 751–805.2013.

11 .Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, and L. K. Nolan.Characterizing the APEC pathotype.Vet. Res. 36: 241–56.2005.

12. Rosario, C. C., a C. C. López, I. G. Téllez, O. a Navarro, R. C. Anderson, and C. C. Eslava.Serotyping and virulence genes detection in Escherichia coli isolated from fertile and infertile eggs, dead-in-shell embryos, and chickens with yolk sac infection.Avian Dis. 48: 791–802.2004.

13. Someya, A., K. Otsuki, and T. Murase.Characterization of Escherichia coli strains obtained from layer chickens affected with colibacillosis in a commercial egg-producing farm.J. Vet. Med. Sci. 69: 1009–1014.2007.

14. Vandekerchove, D., P. De Herdt, H. Laevens, and F. Pasmans.Colibacillosis in caged layer hens: Characteristics of the disease and the aetiological agent.Avian Pathol. 33: 117–125.2004.

15. Wang, Y., C. Tang, X. Yu, M. Xia, and H. Yue.Distribution of serotypes and virulence-associated genes in pathogenic Escherichia coli isolated from ducks.Avian Pathol. 39: 297–302.2010.

Table 1. Combination of genes identified in the virulent strains and their proportion in each genetic line

Pattern	# of Genes	Identified Genes	Total	Line A	Line B
#1	6	All	9	1 4%	8 42%
#2	5	*iroN, iss, hlyF, iutA, ompT*	7	3 12%	4 21%
#3	4	*iroN, hlyF, iutA, ompT*	5	5 20%	0 0%
#4	4	*iroN, iss, hlyF, iutA*	1	0 0%	1 5%
#5	4	*iss, hlyF, iutA, ompT*	1	1 4%	0 0%
#6	4	*hlyF, iutA, ompT, tsh*	1	0 0%	1 5%
#7	3	*hlyF, iutA, ompT*	19	14 56%	5 27%
#8	3	*iss, hlyF, ompT*	1	1 4%	0 0%

MOVING FORWARD: AN ALTERNATIVE MANAGEMENT STRATEGY TO PROTOZOAL INFECTIONS ASSOCIATED WITH ECONOMIC LOSS IN THE POULTRY INDUSTRY

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SUMMARY

Protozoal infections in the poultry industry are among the most important and leading causes of economic losses. The demand for poultry meat that is raised antibiotic free (ABF), no antibiotics ever (NAE) or organic (OR) continues to increase (4, 25, 28). Implementation of the Food and Drug Administration guidance 209, 213 and the Veterinary Feed Directive (VFD) led to removal of growth promoting claims for medically important antibiotics and increased veterinary oversight. In addition, the ban of some anti-protozoal treatment and prophylactic drugs, has led to a re-emergence of morbidity and mortality caused by uncontrolled protozoal infections. As a result, producers are looking for alternative methods to control protozoal infections and reduce losses associated with disease. Plant-derived compounds have been the focus of possible solutions with a higher level of acceptance by consumers than the previously used chemotherapeutic counterparts. The most common plant derivative compounds are based on combinations of essential oils, saponins, phenols and various other plant extracts(15, 17, 21, 23, 24, 27). One of the most common complaints among poultry producers is the variability of outcome when introducing new alternative plant derived products to control protozoal infections. Many of these supplemental alternatives show to be efficacious *in-vitro*, but fail to be successful in field trials, or vice versa(7, 13, 17). Although their mode of action is not completely understood, they continue provide a successful alternative to protozoal disease when used appropriately. Utilization of these programs often requires increased emphasis on husbandry management practices, enhanced biosecurity, adequate down-time between flocks and appropriate vaccination programs. The goal of utilization of these all-natural alternative programs is to enhance the bird's gastrointestinal immune system, enhance repair of the gut epithelium and to reduce the potential production losses associated with protozoal disease.

Dynamic Changes in Poultry Production. In the United States, poultry is the primary source of protein with over 90 pounds consumed per capita. The consumer demand for poultry meat that is raised organic, without antibiotics and no antibiotics ever has increased over the last decade and continues to increase(4, 22, 28). Although in 2012 it was estimated that the number of total meat sales that was ABF, NAE or OR was less than 5%, projections on this demand are estimated to reach closer to 25% over the next few years (14). In part, this increase is from consumer pressure on food suppliers which has led to increase demand of poultry producers.

The poultry industry has been transitioning from the typical conventional systems to meet the increasing demand of ABF, NAE and OR poultry meat products (4, 28). These new programs limit or eliminate usage of antibiotics and chemotherapeutics. However, in the presence of disease, treatment of the birds is necessary, affecting the label for raised organic, ABF or NAE for that flock and potentially subsequent flocks. This creates an increase in financial loses that would not allow producers to successfully continue to raise birds under their respective labels. Increased financial stress and failure to continue to meet customer's labels has led some producers to return to conventional systems of rearing. Therefore, there is great interest to develop and implement a successful disease control programs that rely on alternative natural products.

Common Poultry Protozoal Agents of Economic Loss. In the poultry industry, coccidiosis is the major disease of concern caused by protozoal organisms. Globally, it is estimated that the financial losses due to coccidiosis are more than $3 billion annually (29). In the US, recent poultry industry surveys ranked coccidiosis as the first broiler disease(2) and thirteenth in turkeys (6). Coccidiosis is caused by the apicomplexan protozoan *Eimeria*. The most common chicken infections seen in the field include *E. acervulina, E. maxima, E. tenella* (9, 18). In turkeys, there are four pathogenic species of economic concern, which include *E. adenoeides, E. meleagrimitis, E. dispersa and E. gallopavonis* (5, 18*)*. Clinical signs and disease severity in poultry range from mild to severe. The most damage associated with *Eimeria* spp. occurs during cycling, where invasion of intestinal mucosa induces structural damage, inflammation and increased mucus secretion. Secondary infections such as necrotic enteritis and

septicemia are often seen during this time (30). Damage to the gut leads to other clinical signs such as malabsorption of nutrients, diarrhea, and in serious cases mortality (9, 12, 16, 18).

Although coccidiosis continues to be the primary protozoal agent associated with production loses and decreased feed conversion, another protozoal disease of concern in the poultry industry is Histomoniasis commonly known as Blackhead Disease (BD) (15, 16, 18, 19). In a trending milieu of a list from removed chemotherapeutic agents, recent changes banned the use of arsenic-based compounds used for treatment and prevention of protozoal infections. A rise of other parasitic diseases such as BD has been increasingly more notable. BD is caused by the flagellated protozoal parasite *Histomonas meleagridis*. Poultry can become infected with *H. meleagridis* through ingestion of infected cecal worms or through cloacal drinking (common in turkeys). Alone, *H. meleagridis* does not cause clinical lesions. However, in the presence of bacteria such as *E. coli* the manifestation of classic enterohepatitis was observed (1). This connection suggests that modulation of gut microbiota and promotion of overall gut health can be beneficial to prevent and reduce clinical signs. BD can occur in chickens, turkeys and other gallinaceous species. Turkeys are more susceptible to this disease; however, there is an increase number of recurrent outbreaks commercial pullets (10). In some cases, broiler breeder outbreaks are being noted between time of onset of lay and peak production. Co-infection of coccidia and histomonads can cause severe economic impact due to pronounced clinical disease (20).

Transitioning to Non-conventional and Natural Alternatives. Intense methods of rearing applied in commercial poultry and complicated lifecycles of protozoal parasites, have led to challenges in eradication and control programs. Since the 1940's, management strategies for antiprotozoal coccidia control included the use of different chemotherapeutic agents (18, 23). In conventional rearing, these programs are used in combination, shuttle and rotation programs. Nutritional and vaccine programs can also be utilized in combination with these chemotherapeutic agents and are increasingly being implemented in non-conventional practices (8, 26). The decision for which program to use is influenced by the season, style of production practice and previous product experience.

BD control and prevention relied mainly in the use of antibiotics (Dimetridazole, Ipronidazole, Nitrofurans) or chemicals (arsenical compounds) (3, 7, 11). The increasing concerns for tissue residues and the development of resistant bacteria from antibiotic use has led ban of these compounds for use in food producing animals in the US. Currently, there are no approved antimicrobial treatments for BD. Efforts to decrease the incidence of *H. meleagridis* focus on prevention through implementation of efficacious deworming programs, treatment of litter and increased biosecurity.

As the demand for ABF, NAE and OR labels grow, natural alternative to conventional poultry production programs continue to be high demand. Plant based substances that exert direct and indirect effects have gained the interest and focus of the industry. Due to the complicated nature of the intestinal tract, microbiota, and interacting disease agents like parasites a single plant based substance or alternative alone may not be enough to help in prevention and overall gut health. Often a combination of plant based substances or alternatives are required. A proprietary blend of essential oils, plant extracts, mannan-rich fraction and proteinated minerals have been used to promote good bacteria, build the bird's defenses and maximize intestinal health (21, 9,10). Managing intestinal challenges utilizing other natural plant derivatives, dietary modification, vaccination has also shown promising results; however, *in vitro* tests are not always reflective of *in vivo* studies (7, 17, 27). Mixed reviews on product consistency and efficacy continue to be problematic and further trials and research utilizing a variety of natural product combinations continues.

Conclusion. As the demand for ABF, NAE and OR continues to increase, application of natural solutions should be considered as part of a multifactorial approach. Potential areas for failure include poor management practices, incorrect application of products, concurrent disease pressure and environmental stressors among others. Management strategies to control parasitic protozoal infections in non-conventional poultry production should be consistent, cost effective, holistic, and tailored to specific situations.

REFERENCES

1. Bradley, R. E., and W. M. Reid. Histomonas meleagridis and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. Experimental Parasitology 19:91-101. 1966.

2. Burleson, M. A. Broiler Industry Report. Pending Publication. October 12-18, 2017.

3. Callait, M. P., C. Granier, C. Chauve, and L. Zenner. In vitro activity of therapeutic drugs against Histomonas meleagridis (Smith, 1895). Poultry Science 81:1122-1127. 2002.

4. Castellini, C., C. Berri, E. l. Bihan-Duval, and G. Martino. Qualitative attributes and consumer perception of organic and free-range poultry meat. World's Poultry Science Journal 64:500-512. 2008.

5. Chapman, H. D. Coccidiosis in the turkey. Avian Pathology 37:205-223. 2008.

6. Clark, S. R., and A. Bailey. Current health and industry issues facing the US turkey industry. Proceedings 120th Annual Meeting of the USAHA, Transmissible Diseases of Poultry and Other Avian Species Comittee. Publication pending. 2016.

7. Clark, S. R., and E. Kimminau. Critical Review: Future Control of Blackhead Disease (Histomoniasis) in Poultry. Avian Diseases. 61:281-288. 2017.

8. Dalloul, R. A., and H. S. Lillehoj. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Review of Vaccines 5:143-163. 2006.

9. Davies, S. F. M., L. P. Joyner, and S. B. Kendall. Coccidiosis. Oliver and Boyd LTD., Edinburgh and London. 1963.

10. Esquenet, C., P. De Herdt, H. De Bosschere, S. Ronsmans, R. Ducatelle, and J. Van Erum. An outbreak of histomoniasis in free-range layer hens. Avian Pathology 32:303. 2003.

11. Flowers, A. I., C. F. Hall, and L. C. Grumbles. Chemotherapy of Histomoniasis of Turkeys I. The Value of 1:2-Dimethyl-5-Nitroimidazole in Prevention and Treatment. Avian Diseases:394. 1965.

12. Frade-Negrete, N. J., X. Hernández-Velasco, B. Fuente-Martínez, M. Quiroz-Pesina, E. Ávila-González, and G. Tellez. Effect of the infection with Eimeria acervulina, E. maxima and E. tenella on pigment absorption and skin deposition in broiler chickens. Arch Med Vet 48:199-207. 2016.

13. Grabensteiner, E., D. Liebhart, N. Arshad, and M. Hess. Antiprotozoal activities determined in vitro and in vivo of certain plant extracts against Histomonas meleagridis, Tetratrichomonas gallinarum and Blastocystis sp. Parasitology Research 103:1257-1264. 2008.

14. Greene, C., C. Dimitri, L. Biing-Hwan, W. McBride, L. Oberholtzer, and T. Smith. Emerging Issues in the U.S. Organic Industry. U.S. Dept. of Agriculture, Economic Research Service. June 2009.

15. Hess, M., D. Liebhart, I. Bilic, and P. Ganas. Histomonas meleagridis—New insights into an old pathogen. Veterinary Parasitology 208:67-76. 2015.

16. Kreier, J. P., and J. R. Baker. Parasitic protozoa, 2nd ed. Academic Press, San Diego. 1991.

17. Liebhart, D., P. Ganas, T. Sulejmanovic, and M. Hess. Histomonosis in poultry: previous and current strategies for prevention and therapy. Avian Pathology 46:1-18. 2017.

18. McDougald, L. R. Intestinal Protozoa Important to Poultry. In. Poultry Science Association, United States. p 1156. 1998.

19. McDougald, L. R., and L. Fuller. Blackhead Disease in Turkeys: Direct Transmission of Histomonas meleagridis from Bird to Bird in a Laboratory Model. Avian Diseases:328. 2005.

20. McDougald, L. R., and J. Hu. Blackhead Disease (Histomonas meleagridis) Aggravated in Broiler Chickens by Concurrent Infection with Cecal Coccidiosis (Eimeria tenella). Avian Diseases:307. 2001.

21. Mendel, F., P. R. Henika, and R. E. Mandrell. Bactericidal Activities of Plant Essential Oils and Some of Their Isolated Constituents against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica. Journal of Food Protection 65:1545-1560. 2002.

22. Padel, S., and C. Foster. Exploring the gap between attitudes and behaviour: understanding why consumers buy or do not buy organic food. British Food Journal 107:606-625. 2005.

23. Peek, H. W., and W. J. M. Landman. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. Veterinary Quarterly 31:143-161. 2011.

24. Pop, L., A. Györke, A. F. Tăbăran, M. O. Dumitrache, Z. Kalmár, C. Magdaş, V. Mircean, D. Zagon, A. Balea, and V. Cozma. Effects of artemisinin in broiler chickens challenged with Eimeria acervulina, E. maxima and E. tenella in battery trials. Veterinary Parasitology 214:264-271. 2015.

25. Smith-Spangler, C., M. L. Brandeau, G. E. Hunter, J. C. Bavinger, M. Pearson, P. J. Eschbach, V. Sundaram, H. Liu, P. Schirmer, C. Stave, I. Olkin, and D. M. Bravata. Are organic foods safer or healthier than conventional alternatives? A systematic review. Annals of Internal Medicine 157:348-366. 2012.

26. Sundar, S. B., T. Harikrishnan, B. R. Latha, G. Sarath, and C. T. S. Kumar. Anticoccidial drug resistance in chicken coccidiosis and promising solutions: A review. Journal of Entomology and Zoology Studies. 5(4):1526-1529. 2017.

27. Thangarasu, M., K. Tien-Fen, W. Yueh-Chen, and Y. Wen-Chin. Herbal Remedies for Coccidiosis Control: A Review of Plants, Compounds, and Anticoccidial Actions. Evidence-Based Complementary and Alternative Medicine, Vol. 2016. Article ID: 2657981. 2016.

28. Van Loo, E., V. Caputo, J. R. M. Nayga, M. , Jean-Francois, P. Crandall G., and R. C. Steven. Effect of Organic Poultry Purchase Frequency on Consumer Attitudes Toward Organic Poultry Meat. Journal of Food Science 75:S384-S397. 2010.

29. Williams, R. B. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. In. Pergamon Press, Great Britain. p 1209. 1999.

30. Williams, R. B. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology 34:159-180. 2005.

IN OVO DELIVERED SYNTHETIC SINGLE-STRANDED RNA INDUCES ANTIVIRAL RESPONSE AGAINST LOW PATHOGENIC AVIAN INFLUENZA VIRUS REPLICATION PRE-HATCH

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SUMMARY

Toll-like receptor (TLR) 7 recognizes microbial single-stranded RNA (ssRNA) and activates signaling mechanisms leading to innate antiviral response. The objective of the study are to determine whether *in ovo* delivered synthetic ssRNA induces antiviral response against low pathogenic avian influenza virus (LPAIV) infection in the respiratory tract pre-hatch and observe the correlates of antiviral response mediated by ssRNA. When synthetic ssRNA was delivered *in ovo* at embryo day (ED)18, with a control group receiving sterile phosphate buffered saline (PBS) and a portion challenged with LPAIV via the same route at ED19 while the rest were sampled. We found that *in ovo* delivered ssRNA significantly reduces LPAIV replication in lungs 1 day post-infection correlating with macrophage recruitment in lungs. *In vitro*, macrophages were producing nitric oxide (NO) in response to ssRNA treatment indicating NO as a potential mediator in ssRNA mediated antiviral response against LPAIV replication. *In ovo* delivered ssRNA may be a potential antiviral agent against LPAIV that require further investigation.

INTRODUCTION

The innate immune system, which is known to elicit broader nonspecific protective host responses against invading pathogens, equipped with a range of immune cells. One of the major immune cells involved in the recognition and the elimination of the microbes is the macrophages. The microbial recognition by macrophages is mediated by the pattern recognition receptors (PRRs) including TLRs (1-3). TLRs are indispensable in detecting microbial pathogens such as viruses, bacteria, and fungi due to the presence of molecules that are highly conserved among groups of microbes known as pathogen-associated molecular patterns (PAMPs) (4-7). PAMP-TLR interaction activates intracellular signaling cascades (8) leading to upregulation of inducible nitric oxide synthase (iNOS) (9, 10). The iNOS facilitates production of nitric oxide (NO), a highly reactive and potent antiviral molecule as a part of innate host defense against invading infectious agents (11, 12).

Of the many types of TLRs, TLR7 is the only identified receptor that binds with viral single-stranded ribonucleic acid (ssRNA) or synthetic ligands similar to ssRNA (such as resiquimod, imiquimod, gardiquimod and loxoribine) in birds (10, 13). Induction of innate immune responses using the synthetic ssRNA has been investigated in various animal experiments. It has been shown a potential antiviral activity of synthetic ssRNA against very virulent infectious bursal disease virus infection in chicken in terms of reduced morbidity and mortality (14). Recent studies provide evidence to support the fact that TLR7 activation enhances NO production in mammals (15, 16). However, it is not known in chickens whether activation of TLR7 pathway stimulate antiviral activity against low pathogenic avian influenza virus (LPAIV) infection, whether *in ovo* delivered synthetic ssRNA is capable of eliciting macrophage response, and whether NO involved in the antiviral mechanisms following activation of TLR7 pathway in chicken. Our objectives of this study were to determine whether *in ovo* delivered synthetic ssRNA, resiquimod, could elicit antiviral response against LPAIV infection pre-hatch and then, to investigate the possible mechanisms of innate antiviral response induced by resiquimod.

MATERIALS AND METHODS

In this study, we delivered synthetic ssRNA, resiquimod, *in ovo* at ED 18 with a control group receiving PBS. At ED 19, a portion was infected with H4N6 LPAIV *via* the same route and subsequently the lungs were sampled at ED 20. The samples were homogenized and the supernatants were collected to quantify the live virus particles using standard plaque assay technique. In rest of the eggs at ED19, the lungs were sampled and preserved in optimum cutting temperature (OCT) compound at -80°C. The preserved lung tissues were sectioned and indirect immunofluorescent assay was used to quantify macrophage numbers. Unlabeled mouse monoclonal antibody specific for chicken macrophages, KUL01

(Southern Biotech, Birmingham, Alabama, USA) was used as primary antibody and DyLight® 550 conjugated goat anti-mouse IgG (H+L) (Bethyl Laboratories Inc., Montgomery, TX, USA) was used as the secondary antibody. The fluorescent signals were imaged using an epifluorescence microscope and quantified using Image J software (National Institute of Health, Bethesda, Maryland, USA).

In vitro, the avian macrophages were cultured for 24 hours and stimulated with resiquimod, an iNOS inhibitor, N-([3-(Aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride **(**1400W) (Sigma-Aldrich, St. Louis, MO, USA), resiquimod together with 1400W and growth media only (control). The resultant macrophages culture supernatants were collected at 24 hours post-treatment and a portion was transferred to Madin-Darby Canine Kidney (MDCK) cell culture before inoculation with H4N6 LPAIV. The NO concentration in the remaining culture supernatants was quantified using Griess assay reagent system.

RESULTS

We found that *in ovo* delivered synthetic ssRNA significantly reduces LPAIV replication in lungs 1 day post-infection correlating with increased macrophage recruitment in lungs. *In vitro*, we showed that NO originated from macrophages following resiquimod stimulation are capable of eliciting the antiviral response against H4N6 LPAIV infection.

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

REFERENCES

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

2. Akira, S. Toll-like receptor signaling. The Journal of biological chemistry 278:38105-38108. 2003.

3. Meylan, E., J. Tschopp, and M. Karin Intracellular pattern recognition receptors in the host response. Nature 442:39-44. 2006.

4. Galiana-Arnoux, D., and J.L. Imler Toll-like receptors and innate antiviral immunity. Tissue antigens 67:267-276. 2006.

5. Haynes, L.M., D.D. Moore, E.A. Kurt-Jones, R.W. Finberg, L.J. Anderson, and R.A. Tripp Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. Journal of virology 75:10730-10737. 2001.

6. Lester, S.N., and K. Li Toll-like receptors in antiviral innate immunity. Journal of molecular biology 426:1246-1264. 2014.

7. Arpaia, N., and G.M. Barton Toll-like receptors: key players in antiviral immunity. Current opinion in virology 1:447-454. 2011.

8. Sasai, M., and M. Yamamoto Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors. International reviews of immunology 32:116-133. 2013.

9. Haddadi, S., S. Thapa, A.M. Kameka, J. Hui, M. Czub, E. Nagy, G. Muench, and M.F. Abdul-Careem Toll-like receptor 2 ligand, lipoteichoic acid is inhibitory against infectious laryngotracheitis virus infection in vitro and in vivo. Developmental and comparative immunology 48:22-32. 2015.

10. Abdul-Cader, M.S., A. Amarasinghe, and M.F. Abdul-Careem Activation of toll-like receptor signaling pathways leading to nitric oxide-mediated antiviral responses. Archives of virology 161:2075-2086. 2016.

11. MacMicking, J., Q.W. Xie, and C. Nathan Nitric oxide and macrophage function. Annual review of immunology 15:323-350. 1997.

12. Abdul-Cader, M.S., H. Ahmed-Hassan, A. Amarasinghe, E. Nagy, S. Sharif, and M.F. Abdul-Careem Toll-like receptor (TLR)21 signalling-mediated antiviral response against avian influenza virus infection correlates with macrophage recruitment and nitric oxide production. The Journal of general virology 98:1209-1223. 2017.

13. Philbin, V.J., M. Iqbal, Y. Boyd, M.J. Goodchild, R.K. Beal, N. Bumstead, J. Young, and A.L. Smith Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. Immunology 114:507-521. 2005.

14. Annamalai, A., S. Ramakrishnan, S. Sachan, B.S.A. Kumar, B.K. Sharma, V. Kumar, M. Palanivelu, B.P. Varghese, A. Kumar, B.C. Saravanan, and N. Krishnaswamy Prophylactic potential of resiquimod against very virulent infectious bursal disease virus (vvIBDV) challenge in the chicken. Veterinary microbiology 187:21-30. 2016.

15. Lee, J., N. Martinez, K. West, and H. Kornfeld Differential adjuvant activities of TLR7 and TLR9 agonists inversely correlate with nitric oxide and PGE2 production. PloS one 10:e0123165. 2015.

16. Moisan, J., W. Wojciechowski, C. Guilbault, C. Lachance, S. Di Marco, E. Skamene, G. Matlashewski, and D. Radzioch Clearance of infection with Mycobacterium bovis BCG in mice is enhanced by treatment with S28463 (R-848), and its efficiency depends on expression of wild-type Nramp1 (resistance allele). Antimicrobial agents and chemotherapy 45:3059-3064. 2001.

INVESTIGATING NUTRITIONAL FACTORS INFLUENCING RUNTING AND STUNTING SYNDROME

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INTRODUCTION

Runting and Stunting Syndrome (RSS) is theorized to be a multifactorial disease of chickens caused by various viruses and bacteria, such as rotavirus, reovirus, astrovirus, and parvovirus (1, 3, 4, 5, 6). Clinical signs associated with RSS include diarrhea ("flushing"), subsequently causing wet litter and huddling behavior, stunted growth of individual birds, and abnormal feathering pattern (3). Histopathological lesions of RSS include villus atrophy, dilated crypts, and lamina propria expansion (2). After noting flushing and huddling behavior in Delmarva (DE, MD, VA) broiler flocks consuming feed with questionable ingredient quality, feed was collected from one- to three-week-old flocks and fed back to day-old chicks to determine if the feed was associated with the unexplained enteritis, flushing, and huddling. Two series of studies were conducted in succession, leading to similar clinical signs and histopathological results.

MATERIALS AND METHODS

Feed. Control groups were fed a commercially-available, organic chicken feed from a local farm-supply store. Test groups were fed different samples of feed taken from affected chicken houses with clinical signs of diarrhea and unevenness. Study One contained four groups: Control (C-1); Feed 1 (1-1): conventional feed directly from feed mill; Feed 2 (1-2): caked feed from flushing case; and Feed 3 (1-3): flushing case. Study Two contained two groups: Control (C-2); and Feed 1 (2-1): flushing case.

Chickens. Day-old male Ross 708 chicks were selected from the same flock directly from the hatchery. In Study One, the chicks in groups 1-1, 1-2, and 1-3 were vaccinated via spray vaccine with a commercial coccidiosis and bronchitis vaccines to simulate commercial challenge. Chicks in Study Two were not vaccinated in the hatchery, to remove confounding factors from the coccidiosis and bronchitis vaccines.

Housing. Disinfected metal isolators were used to house the chicks for the two-week duration of the studies. The isolator contained closed, nipple-drinker systems and metal feeders. Flooring was a metal, mesh rack covered in clean brown paper for the first six to seven days to ensure chick comfort and proper coccidiosis cycling. The paper was removed after six to seven days, and birds spent the remaining seven to eight days directly on the metal rack. Feed and water were available *ad libitum*. Each isolator was heated by a single incandescent light bulb for chick comfort. Each group in Study One was housed in a separate isolator. Study Two housed the C-2 and 2-1 group in the same isolator, separating the groups by a clean cardboard wall to expose the birds to an identical environment.

Methods. For Study One, average weights were collected, and fifteen chicks were placed in four separate isolators. Four birds from each group were sacrificed via cervical dislocation at seven days of age, and the following were collected for histological analysis: liver, jejunum (proximal to and including Meckel's diverticulum), proventriculus, gizzard, duodenum, and pancreas. Chicks were left to grow until fourteen days of age and then sacrificed, with the same tissues collected from four more birds. Intestinal contents were collected from the four groups at fourteen days of age for viral detection by polymerase chain reaction (PCR). Study Two was conducted using the same methods as Study One, except that both study groups were housed in the same isolator. Eleven birds were included in group 2-1, and ten in group C-2. Individual body weights were measured at placement, six, and fourteen days, and the first tissue samples were collected at six days of age. Body weights were entered into Microsoft® Excel to calculate averages and percent differences.

RESULTS

At four to seven days of age, groups 1-1, and 1-3 developed signs of RSS, including feed passage, diarrhea, huddling, and uneven sizing. Two birds from group 1-1 were found dead at seven days of age due to apparent feed refusal. Feed presentation was modified, and the birds' eating improved. Adverse clinical signs generally resolved by fourteen days of age, but the body weight differences remained. Histologic examination at seven days of age revealed mild to

moderate multifocal crypt dilation, intraluminal cellular debris, and epithelial attenuation consistent with RSS. Histologic examination at fourteen days of age revealed no signs of RSS. PCR analysis at fourteen days of age revealed negative results for reovirus, astrovirus, rotavirus, and Fowl Adenovirus for groups C-1, 1-1, and 1-3. Group 1-2 was positive for reovirus and astrovirus. The C-1 group had the largest average body weight (344.90 g). Group 1-2 had the second largest average body weight (285.27 g), followed by group 1-3 (203.36 g) and group 1-1 (194.75 g). Groups 1-3 and 1-1 were 41.04% and 43.54% smaller than the control group by weight, respectively.

At four to six days of age, the chicks in Group 2-1 developed huddling, pasty vents, diarrhea, feed passage, and uneven sizing. Chickens in the control group were more even in size and did not exhibit overt signs of diarrhea or stunting at four to six days of age. These clinical signs were generally resolved in individuals by fourteen days of age, however the uneven body weights of the birds remained. Group 2-1 was 28.50% smaller at six days of age and 15.46% smaller at fourteen days of age, with ending average weights of 206.17 g (2-1) and 238.05 g (C-2). Histological examination revealed RSS-like dilation of intestinal crypts in three of the four birds in the 2-1 group at six days of age. One of the four birds necropsied in the C-2 group exhibited mild multifocal crypt dilation, epithelial attenuation, and intraluminal cellular debris in the jejunum, consistent with RSS. Histologic lesions were resolved at fourteen days of age. PCR analysis of intestinal contents at six and fourteen days of age showed that the two groups were positive for reovirus at six and fourteen days of age, but all negative for astrovirus and Fowl Adenovirus. The 2-1 group was positive for rotavirus at fourteen days of age, but not at six days of age.

CONCLUSION

In these studies, the suspect feeds from flushing flocks produced RSS-like signs in chicks housed in isolators. Adverse clinical signs and histopathological lesions were observed shortly after placement, consistent with another study, which likewise found microscopic cystic lesions present at only 24-hours after exposure to RSS litter (3). Furthermore, histopathological findings in the present studies are consistent with lesions of RSS, e.g., villus atrophy, dilated crypts, and lamina propria expansion (2).

Kang *et al*. found that the average body weight of chickens exposed to RSS litter was 30% smaller than the control group (3). Three days after exposure, the difference in weight was significantly different, and five days after starting the experiment, the RSS-exposed group was 50% smaller than the control group (3). The RSS-like groups were 15 to 40% smaller than the non-affected groups in the present studies.

Due to the consistency of the positive PCR results of reovirus at both six and fourteen days, the present studies demonstrate that feed may play a major role in the development of RSS. Awandkar, *et al*. found that reovirus induced cystic dilation of crypts, reduced body weight, and other signs associated with RSS (1). However, reovirus can be isolated from both clinically normal and abnormal chickens (1). Despite mixed results, the trend from these studies demonstrates the multifactorial nature of RSS, and that a general intestinal upset potentially perpetuated by feed may lead to clinical RSS. More in-depth field studies are planned.

REFERENCES

1. Awandkar, et al. Comparative investigations of infectious runting and stunting syndrome in vaccinated breeder chicks by inactivated reovirus and chicks from non-vaccinated breeders. Iranian Journal of Veterinary Research, Vol. 18, No. 1, Ser. No. 58:6-12. 2017.

2. Fletcher, Oscar J and Tahseen Abdul-Aziz. Alimentary System from Avian Histopathology, Abdul-Aziz, Tahseen, et al. 4th ed., pp 321. 2016.

3. Kang, Kyung-Il, et al. Investigation into the aetiology of runting and stunting syndrome in chickens. Avian Pathology, 41(1):41-50. February 2012.

4. Otto, Peter, et al. Detection of Rotaviruses and Intestinal Lesions in Broiler Chicks from Flocks with Runting and Stunting Syndrome (RSS). Avian Diseases, 50:411-418. 2006.

5. Pantin-Jackwood, Mary J. Multicausal Enteric Diseases from 13th edition Diseases of Poultry. Swayne, David E. [ed.], et al. Ames: Wiley-Blackwell: 1322-1325. 2013.

6. Zsak, Laszlo, et al. Chicken Parvovirus-Induced Runting-Stunting Syndrome in Young Broilers. Avian Diseases, 57(1):123-127. 2013.

DEVELOPMENT OF AN OBJECTIVE SCHEME FOR PATHOTYPING AVIAN REOVIRUS

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BACKGROUND

Avian reoviruses have been implicated in viral arthritis in chickens. In the past seven years, it has also been implicated in tenosynovitis/arthritis in turkeys (1). Controlling reoviral infection is mainly done using autogenous multivalent killed vaccines in breeders. The maternally derived antibodies transferred to chicks/poults are protective in the first two weeks of age when young birds are most vulnerable to reovirus infection. After appearance of new reovirus variants in 2011, killed vaccines were developed from the strains isolated from the infected flocks. Serotyping and pathotyping of isolated strains is important before vaccine production. Use of multivalent reovirus killed vaccines is common now in poultry production. Improving the pathotyping scheme is important to differentiate pathogenic from non-pathogenic isolates and to objectively cluster the pathogenic strains based on their virulence.

OBJECTIVES

1. To create overall an objective pathotyping scheme including clinical signs, gross lesions and histopathologic lesions.
2. To correlate the pathotypes with the genetic sequencing data.

Hypothesis. Developing a numerical objective pathotyping scheme including clinical signs, gross and histologic lesions will enable objective clustering of pathogenic avian reoviruses based on the virulence and to correlate with their genetic sequence.

Rational and significance. Since 80%-90% of avian reoviruses are nonpathogenic, it is important to pathotype the identified avian reoviruses. When a reovirus is identified in birds displaying clinical lameness, the pathotype and the serotype of the identified virus should be identified for the purpose of autogenous vaccine production and update. The current pathotyping experimental model needs improvement to fulfill objectivity. Using very broad definitions to describe the gross lesions can help identify pathogenic viruses but will hardly cluster strains objectively based on their pathogenicity. The objective robust pathotyping scheme is very important to rank the pathogenicity of the virus isolates. The proposed robust scheme that relies on multiple criteria will result in a real ranking that is not subjective. Subsequently, the resultant ranking will establish a solid correlation with the genetic sequences. The established correlation will help future reliability of the genetic sequence data for reovirus pathotyping within few days instead of weeks. This will in the future shorten the time required for the autogenous vaccine production.

APPROACH

a. Experimental models: one-day-old chick (SPF)/turkey poults are inoculated with 0.2 mL of median tissue culture infectious dose/ml titer of reovirus via footpad route.

b. Chicks/poults are left for seven days and observed and scored using lameness score (2), gross lesions and hispathologic lesions in gastrocnemius tendon and hock joint (1)

c. Correlation of the pathotypes with the genetic sequences for the future use of molecular pathotyping of avian reoviruses.

PRELIMINARY DATA

Based on the lesion scores developed by Sharafeldin *et al*., 2014, the three turkey arthritis reoviruses (TARV-MN2, TARV-MN4 and TARV-O'Neil) were significantly different in their pathogenicity. By sequencing of M2 gene, the three viruses clustered separately in the phylogenetic tree (3). These data shown in Figure 1 (A and B) indicating the importance of using objective scoring lesion to rank reoviruses on their pathogenicity and correlate this with genetic sequences. Further testing using more isolates will help to establish a solid correlation.

REFERENCES

1. Sharafeldin, T. A., S. K. Mor, A. Z. Bekele, H. Verma, S. M. Goyal, R. E. Porter. The role of avian reoviruses in turkey tenosynovitis/arthritis. Avian Pathol 43:371-378. 2014.

2. Sharafeldin, T. A., S. K. Mor, A. Z. Bekele, H. Verma, S. L. Noll, S. M. Goyal, R. E. Porter. Experimentally induced lameness in turkeys inoculated with a newly emergent turkey reovirus. Vet Res 46: 11-17. 2015.

3. More S. K., D. Marthaler, H. Verma, T. A. Sharafeldin, N. Jindal, R. E. Porter, S. M. Goyal. Phylogenetic analysis, genomic diversity and classification of M class gene segments of turkey reoviruses. Vet Microbiol 176:70-82. 2015
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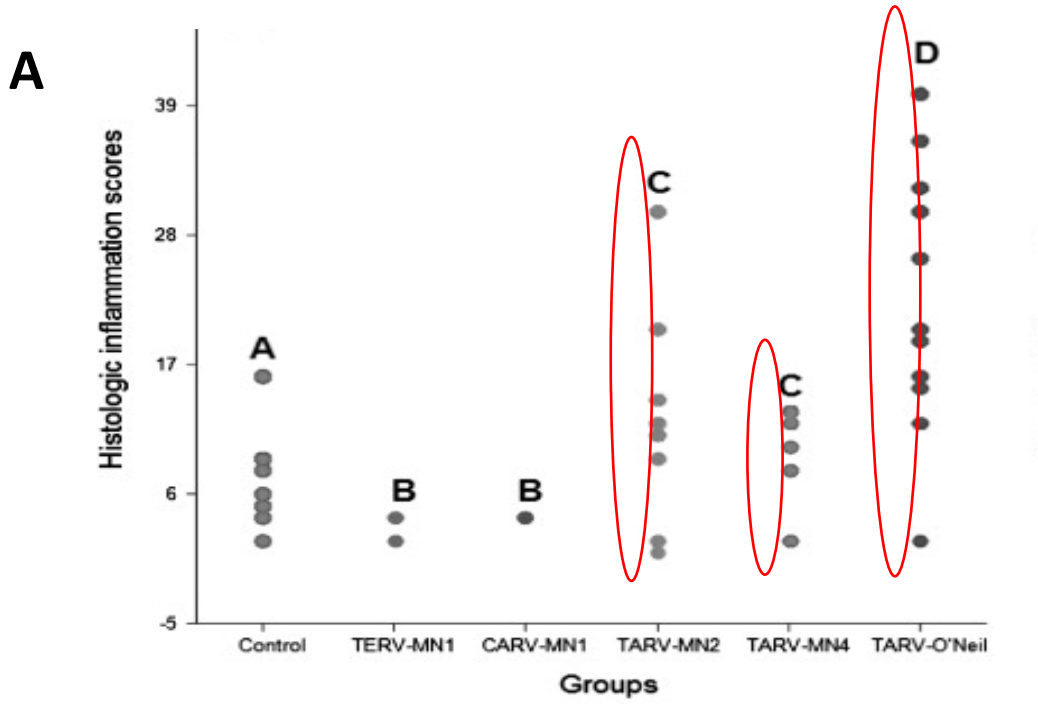
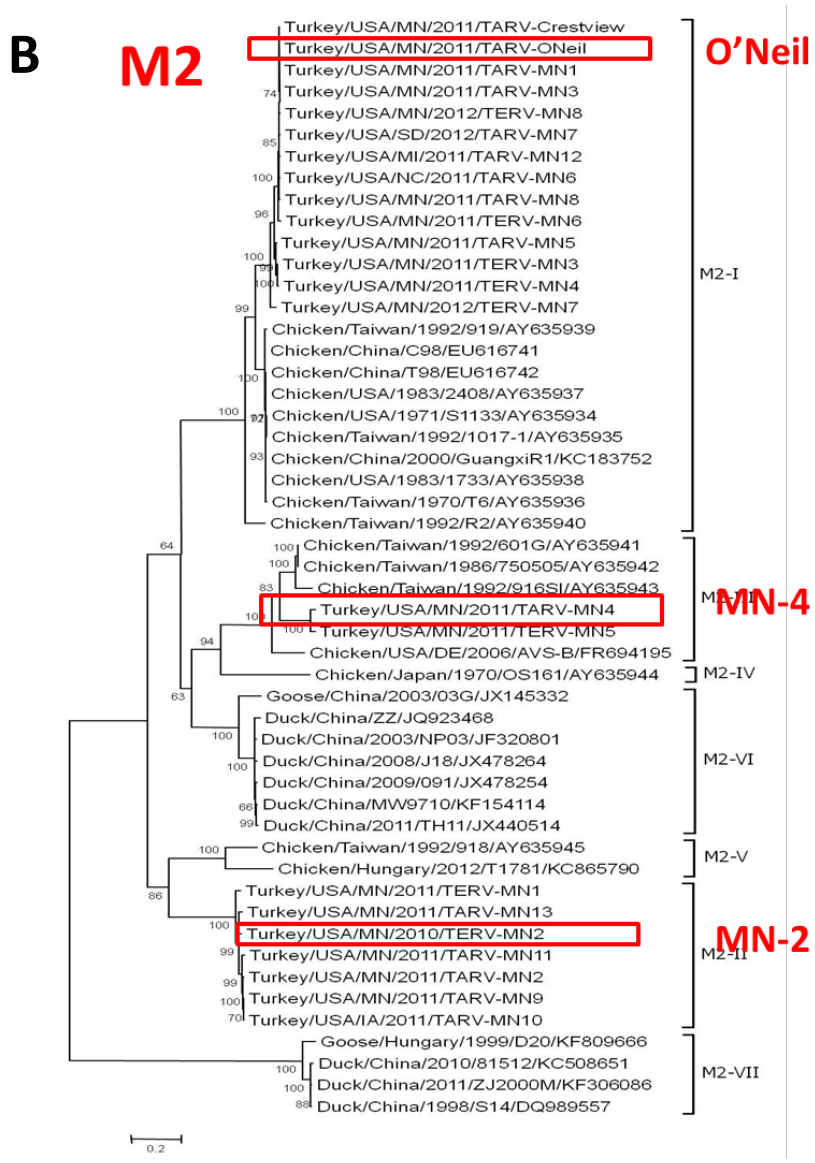
Figure 1. **A**) The histologic inflammation scores of different TARVs was significantly different and significantly higher than turkey enteric reovirus (TERV-MN1) and chicken reovirus (CARV-MN1) that had minimal lesion scores. **B**) TARV-MN2, TARV-MN4 and TARV-O'Neil clustered separately (More than 10% nucleotide sequence divergence) in M2 gene sequence phylogenetic tree.



Sharafeldin *et al*., *Avian Pathology*, 2014



Mor *et al*., *Veterinary Microbiology*, 2015

SUMMARY OF FOWL CHOLERA OUTBREAKS IN COMMERCIAL TURKEYS IN CALIFORNIA, 2007-2017

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SUMMARY

Fowl cholera is a highly contagious infectious disease of various species of poultry caused by *Pasteurella multocida*. Among the species infected, turkeys are particularly susceptible. The disease is characterized by respiratory signs or sudden death in the septicemic form, along with a rapid increase in mortality in the flock. Lesions include polyserositis with enlarged and consolidated lungs, and occasionally enlarged livers and spleens with pale foci of necrosis and inflammation. There are 16 serotypes of *P. multocida*. Serotyping, restriction enzyme analysis, or RFLP of *P. multocida* isolates can be used to study the transmission and epidemiology of fowl cholera in individual company farms or in a region. The electronic data base of California Animal Health and Food Safety Laboratory System was searched for cases of fowl cholera diagnosed in turkeys between 2007 and 2017.

California raises between 15 and 18 million commercial turkeys per year. There were 98 outbreaks of Fowl Cholera in turkeys between 2007 and 2017 involving five commercial companies and 54 different ranches. The disease occurred in both toms and hens equally between the ages of five weeks and 22 weeks, with an average age of 13.6 weeks. Increased mortality ranging from one bird per day to 1200 birds per day with an average mortality of 115 birds per day was the most common clinical complaint. Septicemia characterized by fibrinous airsacculitis, pericarditis, pleuritis, pneumonia with enlarged and consolidated lungs, synovitis, hepatitis and splenitis were the most common lesions observed. *P. multocida* was commonly isolated from lungs, air sac, trachea, pericardium, liver, joints and spleen. Out of the 98 cases where *P. multocida* was isolated, seventy one isolates were serotyped which yielded 17 different serotypes with 3 and 3/4 being the most common. Other serotypes identified included 11, 1, 4, 12, 6, 3/4/14, 4/12/2, 7, *etc.* Seven isolates were not typable. Sixty isolates of *P. multocida* were also fingerprinted and31 different patterns were identified with fingerprint 1340 being the most common (19 isolates), followed by 1341 (6), 1548 (5) and others less frequently.

SEVERE MORTALITY IN A FLOCK OF CHUKAR PARTRIDGES ASSOCIATED WITH PROTOZOA

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SUMMARY

Total mortality ranging from 50.0% to 65.0% by 17-weeks of age were observed in two flocks of 1500 to 2000 Chukar Partridges. Clinical signs included depression, lethargy, reluctant to move and death. Necropsy of six 16-week-old Chukar Partridges revealed enlarged and pale livers and spleens with a few white nodules and segmental distension of ceca with fibrinonecrotic cores. Histopathology revealed hepatitis, splenitis and typhlitis associated with protozoa suggestive of *Histomonas*-like protozoa and amyloidosis. Immunohistochemistry was positive for protozoa suggestive of *Histomonas* in the ceca, liver, spleen, lungs and small intestine. PCR analysis of the livers and cecal contents and molecular characterization suggests the protozoa as a cryptic species of *Tetratrichomonas gallinarum*.

HEMORRHAGIC HEPATITIS ASSOCIATED WITH INCREASED MORTALITY IN BROILER BREEDER REPLACEMENT CHICKS

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SUMMARY

Sudden increase in mortality of 43 to 150 birds per day over a four-day period were observed in a flock of 12,000, four to nine day old broiler breeder replacement chicks. History included birds being off feed and lethargy for a few days. The birds had been vaccinated in the hatchery for Mark's disease, NDV, IBV, ILT and for *Salmonella* Typhimurium. Eleven dead and nine live nine-day-old chicks were submitted for necropsy. Necropsy revealed moderate to severe suffuse to diffuse hemorrhages in the livers and enlarged and congested spleens in of nine of 11 and four of 11 dead chicks respectively. One live moribund chick also had similar lesions in the liver. Histologically lesions in the livers ranged from multifocal necrosis of hepatocytes with hemorrhages with no inflammation to fibrin exudation and infiltration of heterophils randomly scattered throughout. Spleens were very congested with fibrin exudation and no inflammation. Seven of nine live chicks had distension of the small intestine with pale serosa, watery contents in the lumen and cecae in three birds were distended with frothy contents. Microscopically the live chicks had mild to moderate cystic enteritis.

Livers from dead birds were negative for virus by isolation. Livers and intestines were also negative for virus by negative stain electron microscopy. Feed was negative for mycotoxins, heavy metals and salt screen was normal. *Salmonella* Typhimurium vaccine type was isolated from the livers and spleens. The cause of this condition remains undetermined.

VIRAL IMMUNODEFICIENCY: EFFECTS ON INFECTIOUS BRONCHITIS AND CONTROL

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SUMMARY

Chickens in production environments are exposed to multiple stressors and infectious diseases, which impair innate and adaptive immunity (2). Chicken anemia virus (CAV), infectious bursal disease virus (IBDV), and Marek's disease virus are examples of endemic pathogens altering the host's humoral and cell-mediated immune function.

Viral immunodeficiency affects the outcome of infectious bronchitis virus (IBV) infection in chickens and is responsible for failure of adequate protection in vaccinated flocks. Epidemiological and histopathological data on IBV isolations from cases of respiratory disease in broilers aged 27 through 43 days, coinciding with lymphocytic depletion of bursa and/or thymus, provided circumstantial evidence that immunodeficiency and IBV incidence are linked (3). Further experimental evaluations of the effects of immunodeficiency caused by co-infection with CAV and IBDV on outcome of IBV infection confirmed clinical signs and histologic lesions to be more severe and persistent in immunodeficient chickens. At the same time, IBV RNA concentrations in tracheas and lachrymal fluids are higher and more persistent in immunodeficient chickens.

Increased and more persistent viral loads are the result of reduced and/or delayed immune responses in immunodeficient chickens (3). For example, we compared the immune responses to IBV in the Harderian gland and cecal tonsils of immunocompetent chickens and chickens infected with CAV and/or IBDV. Flow cytometry analyses of lymphocytes in Harderian glands and cecal tonsils indicated that the relative abundance of IgM+ B cells in the Harderian glands and cecal tonsils following exposure to IBV in combination with immunosuppressive viruses was reduced compared to chickens infected with IBV alone. CAV, but not IBDV, reduced the CD4+/CD8+ T cell ratios compared to chickens infected with IBV alone. Enzyme-linked immuno-spot forming assays on cells in the Harderian glands and cecal tonsils of IBV-infected chickens indicated that maximum IBV-specific IgA-secreting cell responses were reduced in chickens infected with CAV. IBDV co-infected chickens displayed a delayed IgA response to IBV. Thus, immunosuppressive viruses reduced B cells and T helper cells in response to IBV in the Harderian glands and cecal tonsils, and slowed the kinetics and/or reduced the magnitude of the mucosal immune response against IBV (4).

Another relevant, though less investigated aspect of viral immunodeficiency in chickens, is its impact on evolutionary pathways of IBV. We followed changes in a portion of the S1 gene sequence of the dominant populations of an IBV Ark vaccine strain during serial passages in chickens infected with the immunosuppressive CAV and/or IBDV as well as in immunocompetent chickens. IBV-Ark vaccine subpopulations quickly became extinct after only a few successive passages in immunocompetent birds. In contrast, a distinct IBV subpopulation became established in immunodeficient chickens and persisted through several passages (1).

These results indicate that selection does not cease in immunodeficient chickens, and increased replication cycles of IBV in immunodeficient chickens provides an additional opportunity for emergence of novel IBV. Protection of the immune system by effective vaccination programs as well as maintaining optimum environmental conditions in poultry houses is essential to reduce economic losses from immunosuppressive and associated pathogens.

REFERENCES

1. Gallardo, R.A., V.L. van Santen, and H. Toro. Effects of chicken anemia virus and infectious bursal disease virus-induced immunodeficiency on infectious bronchitis virus replication and genotypic drift. Avian Pathol. 41:451-458. 2012.

2. Hoerr, F.J. Clinical aspects of immunosuppression in poultry. Avian Dis. 54:2-15. 2010.

3. Toro, H., V.L. van Santen, L. Li, S.B. Lockaby, E. van Santen, and F.J. Hoerr. Epidemiological and experimental evidence for immunodeficiency affecting avian infectious bronchitis. Avian Pathol. 35:1-10. 2006.

4. van Ginkel, F.W., V.L. van Santen, S.L. Gulley, and H. Toro. Infectious bronchitis virus in the chicken Harderian gland and lachrymal fluid: viral load, infectivity, immune cell responses, and effects of

viral immunodeficiency. Avian Dis. 52:608-617. 2008.

VACCINES AND SMALL FLOCK POULTRY: YES, NO, MAYBE?

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SUMMARY

Small flock or backyard poultry rearing is an ever increasing hobby, particularly in urban areas. For management and disease control purposes, I have grouped non-commercial poultry into 3 categories: 1) Pet and small personal flocks, 2) Exhibition flocks both breeders and hobbyists, and 3) Small commercial operations for production purposes (meat or eggs). Disease pressure can be significantly different for each of these categories. Most pet flocks have little disease pressure, remain in place, and management is typically the major health concern. Exhibition (including cock-fighting) flocks experience significant exposure to disease with frequent travel to shows / events and often frequent additions to the flock. With small commercial flocks, production demands and rearing categories (organic, antibiotic free, etc.) mimic the disease pressure observed in large commercial flocks.

Marek's disease (MD). All chickens no matter their use must be vaccinated against MD. Currently MD ranks as the number one or two disease observed in small flocks, rotating with coccidia. Frequently birds are obtained from small hatcheries and or "middlemen" such as feed stores. Chicks are frequently not hatchery vaccinated unless the client specifically requests MD vaccine. Many people are unaware of the existence of MD, and are often erroneously informed that it is a disease only found in commercial flocks.

Coccidia. I do not recommend vaccination against coccidia unless the client has chosen to raise organic birds. I discourage pet flock owners from choosing the organic route, and advise them to use medicated feed during growout. Frequently the small commercial flocks are being raised for a niche market - organic, floor, pasture, etc. and in these cases vaccination against coccidia is advised.

Pox. In areas with high mosquito populations and pasture raising or outdoor access of poultry, pox virus vaccination is recommended if a local outbreak occurs. Since pox vaccine can be effectively used in the face of an outbreak, I generally do not recommend vaccination unless exposure is imminent.

***Mycoplasma gallisepticum* (MG)**. Unfortunately, MG is a frequent diagnosis in backyard flocks and MG free specialty breeders are almost impossible to find. When faced with an MG diagnosis, vaccination can prevent further loss in clinically normal flock mates. However, since MG vaccination does not prevent transmission of MG to other birds, I only recommend its use after closing the flock. For breeders (exhibition or small commercial), depopulation and restocking with hopefully clean birds is the only ethical solution. However, MG regulations often only apply to large commercial breeders and indemnity is rarely available for small flocks. Frequently small flock breeders refuse to depopulate and thus MG continues to circulate.

Avian encephalomyelitis virus (AE). I do recommend AE vaccination for breeder flocks. Although not a major issue in small flocks at this time, and my emphasis has always been MD, we are seeing an increase in AE. Generally these birds are purchased from a feed store or other intermediate source so the connection of the AE infection in the chicks is rarely made to lack of vaccination of the hens.

Salmonella. I recommend this vaccination for small commercial flocks or anyone who is selling meat or eggs. This is particularly true for organic flocks.

Infectious bursal disease virus, infectious bronchitis virus, Newcastle disease virus and laryngotracheitis virus (ILT). If any of these four diseases is endemic or exposure risk is high, I recommend these vaccines primarily for small commercial flocks. There have been incidents of ILT in exhibition birds provoking regulators in the area to require vaccination. The risk of mixing ILT vaccinated birds with an unvaccinated population, resulting in further transmission, is extreme. I feel that ILT vaccination needs to be reserved only for incidents where biosecurity and other control methods have failed.

THE COMPOSITION OF GUT MICROBIOTA AFFECTS THE SUSCEPTIBILITY OF NECROTIC ENTERITIS IN CHICKENS

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ABSTRACT

Good quality of gut microbiota is the key to boost innate immunity against various diseases in healthy animals and humans. Necrotic enteritis (NE), caused by *Clostridium perfringens* (CP), is a multifactorial and re-emerging disease in chickens. CP resides in broilers' intestines from a day old to the marketing age. The limited use of growth promoters and anticoccidial drugs have spiked incidences of necrotic enteritis. The gut microbiome influences the following: the integrity of the gut, mucus layer development, absorption of nutrients, and modulation of pro-inflammatory cytokines. We recently analyzed microbiome from the jejunum and cecum of broilers challenged with *Eimeria*, *Eimeria* + CP, or placebo group, indicating that *Lactobacillus* spp., *Bifidobacterium* spp. and several others seem to inhibit the growth of CP in the jejunum of CP-challenged birds; in contrast, *Escherichia*, *Enterobacter*, and *Serratia* seem to promote or result from the growth of CP. The TLR-2 and TLR-4 pathways were triggered by CP challenge and *A. muciniphila* inoculation, respectively. This study suggests that the abundance of *Bifidobacterium* and *Lactobacillus* in the jejunum protects chickens against NE, and alleviates the severity of NE lesions.

INTRODUCTION

Necrotic enteritis is one of the most important enteric diseases in poultry. The disease is characterized by a mass of necrosis and inflammation in the intestinal tract with a significant decline in growth performance and an increase in mortality. The total cost of NE outbreaks is estimated to be $2.6 billion annually worldwide (6). An effective strategy to control *C. perfringens*-associated NE in poultry is using in-feed antibiotics (growth promoters). Nevertheless, the use of antibiotics in animals is being restricted by many countries because of the emergence of antibiotic-resistant pathogens to humans. With a ban of in-feed antibiotics, the incidence of NE has increased in broiler farms (1). Finding an alternative strategy to substitute the use of growth promoters seems to be imperative, evidenced by increased research on prebiotics, probiotics, and immunity-booster compounds.

Several studies have shown that a healthy gut is positively correlated with the presence of beneficial bacteria; the healthy gut is negatively correlated with the presence of pathogens (4, 5). However, the impact of microbiome on the susceptibility or resistance of necrotic enteritis remain unclear. Therefore, our central hypothesis was that necrotic enteritis is an imbalanced gut microbiome disease, and the composition of gut microbiota affects the outcome of the disease.

The specific objectives of this proposal were to evaluate the effectiveness of lauric acid and *Akkermansia muciniphila* (AM) against necrotic enteritis, and understand the relationship among CP, cocci and other microbiome in the jejunum.

MATERIALS AND METHODS

Necrotic enteritis challenge model. Broilers were divided into eight groups and were fed a wheat diet from day one to eight. From day eight, all groups were fed wheat diets supplemented with 50% fishmeal (w/w). Lauric acid (4g/kg) in feed was given from day eight to the end of the trial. At day ten, all groups, except the control group, CP only and CP+AM groups, were treated with a 10-fold cocci vaccine via an oral gavage. At day eight to 18, the AM group was given *A. muciniphila* at a dose of 10^9 CFU/bird. At days 15, 16, 17 and 18, the CP challenged groups were given *C. perfringens* at a dose of 7.5 x 10^8 CFU/bird three times per day. Intestinal contents and jejunum tissues were collected at day 19 and lesion scores were recorded.

Metagenomic sequencing. DNA was isolated from intestinal contents using a PowerFecal® DNA Isolation Kit (Qiagen) and amplified using universal primers (F338, CCTACGGGNGGCWGCAG; R806, GACTACHVGGGTATCTAATCC) that target the V3~4 regions of bacterial 16S rDNA. Amplified DNA samples were barcoded, pooled to construct the sequencing library, and sequenced using an Illumina Miseq (Illumina) to generate pair-ended 300 × 300 reads.

RESULTS AND DISCUSSION

NE challenge results. The typical NE gross

lesions with focal or multifocal necrosis in the small intestines were observed in 10% of the CP challenged group, 20% of the *Eimeria* + CP group, 30% of CP+ *Eimeria* + lauric acid group, and 40% of the CP+ *Eimeria* + AM group. But, no NE lesions were observed in the *Eimeria*+ AM, *Eimeria* only, and the negative control group. Histological images confirmed the presence of CP and *Eimeria* organisms in jejunum and ileum tissues in the CP- and/or *Eimeria*-challenged groups. The side-by-side NE challenge model, with or without cocci, is proven to be useful.

Mucin, tight junction, and toll-like receptor pathways. muc2 mRNA was lower in the challenged birds than the control birds that is consistent with other studies (2, 3). mRNA levels of tight junction (occludin and claudin 1) were not significantly different between the challenged birds and the control but the mRNA levels decreased in thee challenged birds. TLR-2 and TLR4 pathways were triggered by the CP and AM challenges, respectively.

Microbiota results. The microbial β-diversity between jejunum and cecum samples in all groups were separated into two distinct clusters. The distribution of the most abundant bacterial phyla was more diverse in jejuna than in ceca. The *Eimeria*, CP and AM inoculations significantly altered gut microflora composition, evidenced by heat map and principal coordinate analyses. Interestingly, *A. muciniphila*, a mucin-degrading bacterium, enhanced both the growth and/or the virulence of *C. perfringens*. The abundance of *Lactobacillus* and *Bifidobacterium* was negatively correlated with necrotic enteritis, suggesting that probiotic bacteria can inhibit the growth of CP and reduce the severity of necrotic enteritis in chickens (Figure 1).

REFERENCES

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

2. Golder HM1, Geier MS, Forder RE, Hynd PI, Hughes RJ. Effects of necrotic enteritis challenge on intestinal micro-architecture and mucin profile. Br. Poult. Sci. 52:500-6. 2011.

3. Kitessa SM, Nattrass GS, Forder RE, McGrice HA, Wu SB, Hughes RJ. Mucin gene mRNA levels in broilers challenged with eimeria and/or *Clostridium perfringens*. Avian Dis. 58:408-14. 2014.

4. M'Sadeq, S. A., S. Wu, R. A. Swick, and M. Choct. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics plasing-out worldwide. Anim. Nutri. 1:1-11. 2015.

5. Neumann, A. P., G. Suen. Differences in major bacterial populations in the intestines of mature broilers after feeding virginiamycin or bacitracin methylene disalicylate. J. Appl. Microbiol. 15:15-26. 2015.

6. van der Sluis, W. Clostridial enteritis is an often underestimated problem. World Poult.16:42-43. 2000.

Figure 1. Dot plots depict relative abundance of *Bifidobacterium* and *Lacotbacillus* in various treatment groups. *Represents $p<0.05$ by a one-way ANOVA with Tukey's test; **represents $p<0.01$