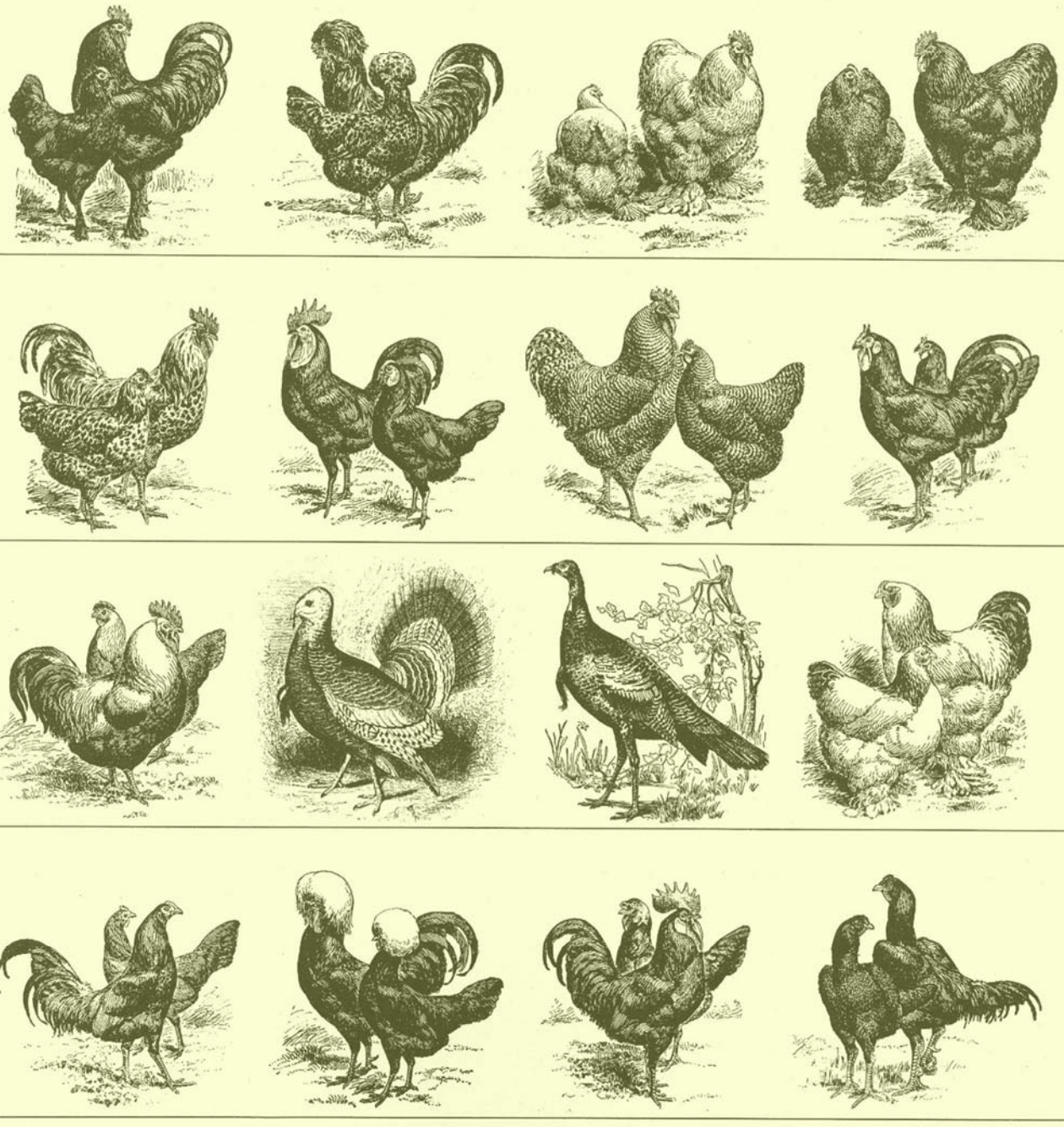
PROCEEDINGS OF THE SEVENTY-THIRD WESTERN POULTRY DISEASE CONFERENCE

April 15-17, 2024 Salt Lake City, Utah

WESTERN POULTRY DISEASE CONFERENCE



PROCEEDINGS OF THE SEVENTY-THIRD WESTERN POULTRY DISEASE CONFERENCE

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73rd WESTERN POULTRY DISEASE CONFERENCE

DEDICATION

DAVID D. FRAME, DVM, DACPV

Dr. David Frame has been an instrumental part of the Western Poultry Disease Conference (WPDC) since 1985. To honor and thank him for his many years of service, the WPDC Board of Directors is dedicating the 73rd WPDC to Dr. David Frame.

Since becoming the Proceedings Editor for WPDC in 2000, Dr. Frame has spent many hours on the WPDC Proceedings, evolving the accessibility and quality of the proceedings by moving them from print copies to CD and flash drives and now online. The knowledge, support, and expertise he has brought to the conference over the years has been invaluable, and all those involved are grateful to have learned from him. Looking back over past WPDC Proceedings, it is clear Dr. Frame has had some memorable WPDC conferences!

Not only has he been a major contributor to WPDC, receiving the 67th WPDC Special Recognition Award in 2018, but has been recognized in industry receiving the Service Appreciation Award (2004, 2005 and 2006), as well as the Scientist of the Year award by the Pacific Egg & Poultry Association in 2016.

Born and raised in Kearns, Utah, he became interested in birds at a young age, designing a breeding and growing program for exhibition chickens. Judging these birds is still a task he continues to do to this day.

In 1980, he completed his Bachelor of Science (BS) in Animal Science from Utah State University (USU), then his Doctor of Veterinary Medicine (DVM) in 1984 from Oregon State University.

Thereafter he completed at two-year residency in avian medicine at the University of California, Davis and obtained diplomate status in the American College of Poultry Veterinarians (ACPV) in 1992. After his residency, he returned to Utah as the Chief Veterinarian for Moroni Feed Company, focusing on breeder and meat turkey health and diagnostics for the next 12 years.

After his time at Moroni Feed Company, he accepted a position as Extension Poultry Specialist at USU, a position he held until his recent retirement in late 2023. At USU, Dr. Frame supported and educated all sectors of poultry, including backyard, game and commercial. He was also an associate professor in the Department of Animal, Dairy and Veterinary Sciences at USU as well as Avian Veterinarian for USU's Central Utah Veterinary Diagnostic Laboratory.

The 73rd WPDC is honored to dedicate this year's meeting to Dr. David Frame, and thanks him for all his contributions, mentoring and expertise.

IN MEMORIAM
STEWART JOHN RITCHIE
1958 – 2023

Stewart John Ritchie passed away on May 2nd, 2023, at home with his much-loved family and dog at his side. Stew was born in Vancouver, BC to Bill and Maud and spent his early years with his 3 siblings in Burnaby, BC before moving to Abbotsford, BC in the 1960s. Stew was a curious and spirited kid who developed a deep respect and love for animals at a young age. While living in rural Abbotsford and under the influence of his father, a hands-on leader in animal feed industry, Stew tried his own hand at raising every kind of farm animal that his parents allowed. Stew delved into the welfare and nutrition aspect of animal husbandry in his teens raising beef cattle, pigs, sheep and chickens. This of course set the stage for a career path that he would follow for the rest of his life.

Stew met his wife Sandra in high school. They spent 48 exciting years together. During this time Stew completed his Bachelor of Agriculture Science at the University of British Columbia (1980), Master's Degree in Animal Science at the University of Arkansas (1982) and Doctor of Veterinary Medicine (1987) at the Western College of Veterinary Medicine in Saskatoon, SK. After a year of travel with Sandra, Stew returned to his hometown of Abbotsford to join his mentor Dr. Doug McCausland in his veterinary practice focusing on poultry and swine. Stew eventually bought Dr. McCausland's practice and founded Canadian Poultry Consultants Ltd. Over the next 30+ years, Stew became a leader in his field, establishing a poultry veterinary consultant practice, followed by a research farm with a focus on improving broiler health and performance. He was passionate about collaboration with his wide network of esteemed peers and created platforms for other experts in the fields of poultry diseases to meet and share their research expertise.

Many may remember Stew for being such an awesome host at the 54th WPDC (2005) held in Vancouver, BC. while he served as Program Chair. He set the entertainment bar so high that subsequent program chairs have been left in the dust.

Stew traveled the world as a consultant in poultry disease management/ prevention and served as an adjunct professor at the University of Georgia and the University of Arkansas. Stew also served as a board member and President of the American Association of Avian Pathologists. In recent years Stew has focused on farming sustainability in broiler production and conveying the essentials of broiler farming to existing and new farmers with his Platinum Brooding Program.

IN MEMORIAM
ARTHUR ALTON BICKFORD
1936 – 2023

Dr. Arthur A. Bickford passed away at home in Turlock on July 29, 2023. He was born and raised on a Vermont dairy farm. In 1960 he received his VMD degree at the University of Pennsylvania. He received his M.S and PhD degrees in 1964 and 1966 respectively, after completing graduate studies at Colorado State University and Purdue University.

Art held faculty positions at Purdue University, the University of Missouri, Columbia, and the University of California, Davis. During his career at the University of California, Davis, Art served in varied capacities, including Extension Veterinarian, Director of Veterinary Extension, Professor of Clinical Diagnostic Avian Pathology in the California Animal Health and Food Safety (CAHFS) Laboratory System, Chief of the Turlock Laboratory, Interim Chief of the Fresno Laboratory, and Associate Director of the Laboratory System. Shortly before his death, he received a special tribute: In his honor, the CAHFS Turlock Branch Laboratory was named the "Arthur A. Bickford-Bruce R. Charlton Turlock Branch Laboratory."

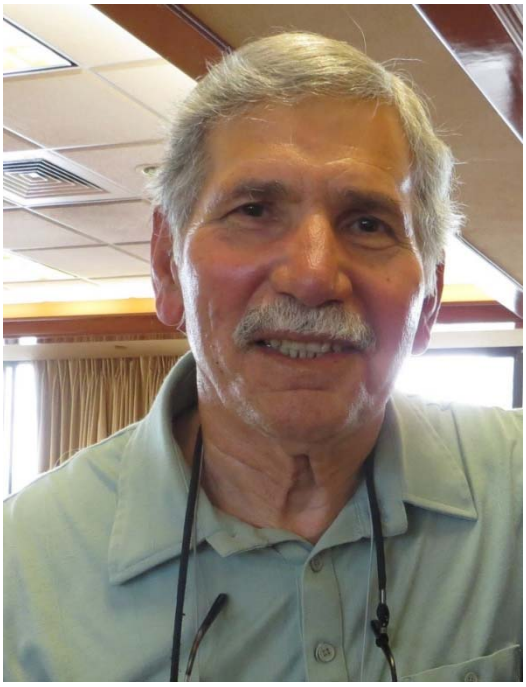
Many honors and awards have been bestowed on Art for his professional expertise and service. Perhaps two among the most notable were the CA Bottorff Award from the AAAP in 1995 and the E. P. Pope Award from the AAVLD in 2001. He was also inducted into the AAAP Hall of Honor in 2016. Art served as the Program Chair (1978) and President (1979) of the 27th and 28th Western Poultry Disease Conferences. He received the WPDC Special Recognition Award in 2001.

Dr. Bickford was a great proponent of the avian residency program. He was fiercely loyal to residents willing to try their best and was readily available to help them as they learned the ropes of poultry diagnostics. Many residents can be proud that Art Bickford is listed as a significant co-author on many of their first published scientific papers. Art, along with Gregg Cutler, Foster Farms, and NuCal Foods established a projected $1 million endowment for the avian medicine residency program. The endowment currently lacks only $80,000 to be fully funded. It is administered through CAHFS and is entitled the "Arthur A. Bickford Endowed Avian Residency Program Fund." Its purpose is to ensure that the avian residency program will be funded in perpetuity for generations of residents to come. Contributions can still be made by contacting the UCD School of Veterinary Medicine Office of Development.

Art and his wife Peggy loved their family, and were very proud of their three daughters, grandchildren, and great-grandchildren. He was at his happiest when talking about them or being involved with their lives.

Dr. Bickford will be most remembered for his wit and common sense approach to solving problems. Also, at least a couple of former residents will remember him for his shameless addiction to a warm piece of good blueberry pie.

IN MEMORIAM
GALESTAN YAN GHAZIKHANIAN
1937 – 2023



Our good friend and colleague, Yan Ghazikhanian, passed away on July 29, 2023. He spent the last years of his life living at his home with his wife Cheryl in Sonoma, California. Dr. Yan was known around the globe for his expertise in turkey health. Yan spent 33 years with Nicholas Turkey Breeding Farms and traveled the world consulting with clients. He was inducted into the AAAP Hall of Honor in 2016. His many achievements and recognitions in the industry are numerous and have been well documented elsewhere.

Yan will be remembered for his generosity, boundless enthusiasm, and quick wit. Sometimes it required a tremendous amount of mental focus to keep up with what he was saying. He was a great teacher and willingly shared his experiences and vast knowledge with others. His dedication and enthusiasm for his work inspired colleagues to do better and try harder.

Yan participated regularly in the WPDC and served as Program Chair for the 29th Western Poultry Disease Conference held jointly with the V ANECA meeting in Acapulco, Guerrero, Mexico in 1980. He subsequently served as WPDC President in 1981. The WPDC acknowledged Yan's many contributions by honoring him with the 2004 Special Recognition Award.

We will miss his wisdom, advice, and expertise; but mostly, we will miss his infectious enthusiasm and friendship.

IN MEMORIAM
LYNN GOODWIN BAGLEY
1957 – 2024

We are saddened by the sudden passing of Dr. Lynn G. Bagley on January 24, 2024 in Storm Lake, Iowa. Lynn loved working with turkeys, and held professional positions in various commercial turkey companies in the US and Canada during his career. He was well-respected throughout the world for his expertise in turkey reproductive physiology. He was a sought-after speaker for many professional meetings.

Lynn was raised in Moroni, Utah, a small mountain valley in the central part of the Beehive State. He grew up growing turkeys with his family. He was the son of Royal and Iris Bagley. His father, Royal, was employed as the veterinarian and hatchery manager of Moroni Feed Company for many years. Lynn earned a bachelor's degree from Utah State University, master's degree from Brigham Young University, and his Ph.D. from North Carolina State University.

Although he thoroughly enjoyed working in all aspects of turkey production, Lynn's greatest love and devotion was to his family and church. As a young man he served a mission for the Church of Jesus Christ of Latter-day Saints in Toronto, Canada and has subsequently served in various local church leadership roles. He married his sweetheart Marilyn Robertson on August 14, 1980. Together they have five children and 17 grandchildren.

Lynn served as Program Chair for the 70th Western Poultry Disease Conference in 2020, a difficult time when the WPDC meeting was held virtually because of the ongoing COVID-19 pandemic. Dr. Bagley was well-known for his friendliness and willingness to share whatever information he could. Keeping in contact with friends and former colleagues was very important to him. Right up to the time of his passing, he was adamant about having monthly virtual "lunch meetings" with a group of poultry colleagues still living in Utah.

73rd WPDC CONTRIBUTORS LIST

(As of April 1, 2024)

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SPECIAL ACKNOWLEDGEMENTS

The 73rd Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and help defray some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international meeting. Many companies and organizations have once again given substantial financial support, including some that also send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

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

Shelly Popowich, Program Chair of the 73rd WPDC, would like to thank the WPDC Foundation Board of Directors, namely Drs. Rodrigo Gallardo, Ana da Silva, Nancy Reimers, Simone Stoute, Carmen Jerry, and David Frame for their support and assistance with this year's meeting. Additionally, a thank-you to all invited speakers as well as graduate student and volunteer moderators.

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

We thank Dr. David Frame for editing and producing another outstanding Proceedings of this meeting. Dr. Frame is indebted to Mr. Dana Frame for formatting of 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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David D. Frame
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73rd WPDC PROCEEDINGS

Please note that the proceedings of the 73rd 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 73rd WPDC, along with past years' proceedings are available in electronic format only. They can be downloaded at https://www.wpdcfoundation.org/past-wpdc-proceedings.

WESTERN POULTRY DISEASE CONFERENCE (WPDC) HISTORY

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

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

YEAR	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
63rd WPDC – 2014	P. Cortez	Ernesto Soto	Hugo Medina	
39th ANECA	Néstor Ledezma M.	Ernesto Soto	Benjamin Lucio Martínez	
64th WPDC – 2015	Ernesto Soto	Shahbaz Haq	Bruce R. Charlton	David Willoughby
65th WPDC – 2016	S. Haq	Susantha Gomis		
66th WPDC – 2017	S. Gomis	C. Gabriel Sentíes-Cué	Richard McCapes	Peter Woolcock Richard P. Chin
67th WPDC – 2018	C.G. Sentíes-Cué	Rodrigo A. Gallardo		David D. Frame
68th WPDC – 2019	R. Gallardo	Sarah Mize		Gregg J. Cutler
44th ANECA	Ricardo Cuetos Collado	Maritza Tamayo		
69th WPDC – 2020	S. Mize	Simone T. Stoute		Mark C. Bland
70th WPDC – 2021	S. Stoute	Lynn G. Bagley	Walter F. Hughes	H. L. Shivaprasad
71st WPDC – 2022	S. Stoute	Simone T. Stoute		
72nd WPDC – 2023	S. Stoute	Carmen Jerry	Peter Woolcock	Charles Corsiglia
73rd WPDC - 2024	C. Jerry	Shelly Popowich		

MINUTES OF THE 72nd WPDC ANNUAL BUSINESS MEETING

President Dr. Simone Stoute called the meeting to order on Monday, March 13, 2023, at 3:30 PM. The meeting was held at the Diablo/El Dorado/Fresno/Granada/Hermosa room, Holiday Inn Downtown Sacramento, CA. All membership was invited and 14 people were in attendance and signed in. Dr. Stoute thanked Dr. Carmen Jerry for her work as Program Chair at this years' WPDC.

APPROVAL OF 71st WPDC BUSINESS MEETING MINUTES

Secretary-Treasurer Dr. Rodrigo Gallardo made a motion to approve the minutes of the 71st WPDC business meeting. Program Chair Dr. Carmen Jerry seconded the motion, and the minutes were approved.

ANNOUNCEMENTS

President Dr. Simone Stoute acknowledged all the contributors, namely Super Sponsors (CEVA and Huvepharma), Benefactors (American Association of Avian Pathologists Inc., Elanco Animal Health, Hygieoa Biological Lans and Zoetis), Patrons (Boehringer Ingelheim, Cobb Vantress Inc., Cutler Associates International, IDEXX, Laboratorio Avimex, SA de CV and Merck Animal Health), and Donors (Alltech, AVS Bio, Diamond V, Hidden Villa Ranch/Nest Fresh, Phibro Animal Health and Veterinary Diagnostic Pathology, LLC). All contributors were acknowledged for their generous support. Dr. Simone Stoute acknowledged WPDC committee of Drs.Carmen Jerry, Rodrigo Gallardo, Nancy Reimers, David Frame, and Ana da Silva as well as WPDC staff Bob Bevans-Kerr and Channah Pool for their administrative support.

REPORT OF THE SECRETARY-TREASURER

Dr. Rodrigo Gallardo presented the Secretary-Treasurer report. Due to the transition to a non-profit organization, the Wells-Fargo account contains $161,013.72 and the UCD account $76,231. The UCD account will be used first for expenses to use up the funds and close the account. The BK contributions (committed) plus registrations (197 total, 181 paid) totals approximately $73,360. The total contributions received is $22,100. Hotel expenses of $15,000 have been paid, with a remaining $38,700 remaining to pay. Drs. Rodrigo Gallardo and Nancy Reimers have been working on the set up and dissolution of the new Wells-Fargo and UCD accounts respectfully.

REPORT OF THE PROCEEDINGS EDITOR

Dr. David Frame presented the Proceedings Editor report. Manuscripts were submitted for the first time through Oxford, a new experience but much needed. There were 64 papers approved and presented. The proceedings can be found on the WPDC Foundation website, or by the QR code located on the program.

REPORT OF THE PROGRAM CHAIR

Dr. Carmen Jerry presented the report of the Program Chair. The new Oxford submission system worked well and Ms. Channah Pool was thanked for her help and support. Of the 84 submitted titles, 6 were rejected due to lack of content or scientific merit.

REPORT OF THE STUDENT PROGRAM CHAIR

Dr. Ana da Silva presented the Student Program Chair report. This is the second year of the student competition. There were two competition categories, Case Reports with 7 students competing and Basic Research with 5 students competing. The winner of each category is to receive $500.With few students submitting presentations, more engagement is needed with principal investigators//supervisors and students to encourage attendance. More information to be sent to student specific associations for next year, as well as to encourage more students from the west to attend.

REPORT OF THE CONTRIBUTIONS CHAIR

Dr. Nancy Reimers presented the Contributions Chair report. The sponsors and contributors were all thanked. Due to WPDC now a nonprofit organization, there will be a minor transition for sponsors to get WPDC Foundation into their system, rather than UCD. Any leads for contributors are welcomed.

FUTURE MEETINGS

2024: 73rd WPDC and ACPV sponsored workshop: Salt Lake City, UT
2025: 74th WPDC and ACPV sponsored workshop: Calgary, AB
2026: TBD

CONTINUING EDUCATION

Continuing education credits will be provided by ACPV, RACE certification. Approval for 20 hours of RACE CE (ACPV will sponsor those who haven't submitted state licence information).

NOMINATION AND ELECTION OF PROGRAM CHAIRS

Shelly Popowich was nominated as the 73rd WPDC Program Chair elected. Dr. Ana da Silva was nominated for Program Chair for the meeting in 2025, nominated by Dr. Rodrigo Gallardo and second by Dr. Simone Stoute. The nominations were closed, and the vote was unanimous for Dr. da Silva.

NEW BUSINESS

To preserve past proceedings, Dr. Rodrigo Gallardo will recruit students and hire them to scan proceedings to be uploaded onto the website.

ADJOURNMENT

Dr. Simone Stoute turned over the presidency to Dr. Carmen Jerry. Dr. David Frame moved that the meeting be adjourned. Adjourned at 4:07 PM.

THE ARNOLD S. ROSENWALD LECTURE

David D. Frame
2024

WESTERN POULTRY DISEASE CONFERENCE

EXPERIENCES OF THIRTY-NINE YEARS AS A POULTRY VETERINARIAN (OR "WHERE DID ALL THAT TIME GO?")

David D. Frame

I am deeply honored and humbled to present the 2024 Arnold S. Rosenwald Lecture. Rosy's career as an Extension Poultry Pathologist and veterinarian can be viewed as the archetype of how cooperative extension interacts with production agriculture.

Rosy was very influential in my life, especially regarding my involvement in the Western Poultry Disease Conference (WPDC) both as a presenter and as a member of the committee. I have fond memories of many evening phone calls with Rosy planning for speakers and preparing agendas for the upcoming meeting. He was always kind and thoughtful toward my wife and me and treated us with the utmost respect. His dining room table was eternally piled with a huge stack of WPDC correspondence (except during his traditional WPDC welcoming parties when a large punch bowl took its place!). After one overnight visit at his Davis home, he took me to the airport for my flight back to Salt Lake City laden with a sack full of persimmons and pistachios. They came in very handy after landing in Salt Lake as that was all I had to eat while waiting for a delayed ride home to Ephraim! I was only one of many, many others who benefitted from his kind generosity.

My presentation will not follow the typical format encountered at the WPDC. Instead, it is more of a stroll down "memory lane," mostly for my own benefit; however, I invite others to join and perhaps glean a morsel or two from my unique experiences as a poultry veterinarian from Utah. For all intents and purposes, my entire career has been extension-associated: taking vital information to the people and helping them solve problems – some very serious and complicated. This was carried out either in the capacity of a company veterinarian or as a university faculty member.

Even in high school I had a goal of becoming a veterinarian – not just any veterinarian, however, but a *poultry* veterinarian. During undergraduate work at Utah State University (USU) I took every opportunity to work at the university poultry farm as a side job. Even though USU did not offer a poultry science degree, I eagerly took all poultry-related animal science classes available as well as every special poultry nutrition and husbandry class that the faculty could legally gin up for me. (I was the only student in the department at the time interested in poultry.)

After graduation, I had the privilege of attending veterinary school at Oregon State University (OSU). The highlight of my time spent at OSU was getting to know two great individuals: Don Helfer and Eva Pendleton. I am forever indebted to them for their interest in me as I pursued a career in poultry. Also during veterinary school, I was able to spend valuable time during externships with Yan Ghazikhanian and Barry Kelly at Nicholas and Royal Bagley at Moroni Feed Company.

Later, because of the gracious efforts of Eva and others unbeknown to me, I was given the opportunity to join a poultry medicine residency program with the University of California, Davis. There I had the privilege of working with and being tutored by great people such as Dick McCapes, Dick Yamamoto, Ray Bankowski, Art Bickford, and George Cooper. The time spent there prepared me well for my subsequent career in industry and extension.

As I was finishing up my residency, I received a call from the CEO of Moroni Feed Company (MFC) asking if I would be willing to come work for them. The Utah turkey industry was battling serious problems with avian influenza (AI) at the time and needed additional veterinary help. After looking over the offer, Lisa and I packed up the family and moved to a remote valley in central Utah. Little did we know we would be raising our family and spending the rest of our lives there (up to now, anyway). This initial baptism by fire struggle with a 1984-85 AI turkey problem was only the beginning. It seems most of my career has been plagued with grappling with some aspect of field outbreaks of AI.

At MFC, I served as chief veterinarian for twelve years working with growers in the Sanpete Valley of Utah. I also serviced our breeder flocks in various locations in the state and worked on quality assurance issues at the feed mill and processing plant. It was an enjoyable job. As Moroni Feed was an integrated cooperative, I had the privilege of being exposed to a variety of interesting challenges dealing with almost all aspects of commercial turkey production.

In 1998 I accepted a faculty position with the Animal, Dairy, and Veterinary Sciences Department at Utah State University. My initial assignment was supervisor of the Utah Agricultural Experiment Station turkey facility located in Ephraim, Utah. Along with my staff and fellow poultry scientists we conducted applied research that was specifically directed at benefitting the commercial turkey producers of Utah. Later, in about 2008 I was transferred to the USU Central Utah Veterinary Diagnostic Laboratory (CUVDL) with a faculty role of Extension Poultry Specialist and poultry diagnostician.

This ability to fully work in extension opened up the opportunity to expand my services to additional aspects of poultry and gamebird production in Utah and other areas of the Intermountain West. My assignment at the CUVDL also provided me with a large array of poultry diagnostic experiences that augmented my extension role.

The following are some of the interesting things I experienced as a poultry veterinarian that I consider highlights of my career even though they may seem mundane or commonplace.

The Orlopp turkey strain used by MFC during the time I was employed by the company was not MM-free. This afforded me the unfortunate opportunity to become very familiar with the lesions and physiologic consequences of this infection. Newly-hatched poults infected with MM often exhibited a peculiar airsacculitis that was distinctly recognizable with a little experience. As the infected turkeys grew, the deformed tibiotarsal joints typical of MM became evident. This is a turkey disease that hopefully most young veterinarians never have to witness in their career.

Infections with *Salmonella arizonae* associated with its typical lesions were also unfortunate findings frequently encountered in young poults during my early career. This may be at least partially attributable to the locations where many of our multiplier flocks were raised. These turkeys were located in areas that frequently favored exposure to reptiles and other wild animals. It has been years since a case of arizonosis has crossed my necropsy table. Maybe I'm just fortunate not to see it anymore.

During my first week on the job with MFC I was called out to a ranch that was having a severe outbreak of "move out enteritis," as the local growers called it. To my astonishment there were a number of dead eight-week-old hens with diarrhea and blood-filled intestines. Histopathologic evaluation later showed typical adenoviral inclusions compatible with hemorrhagic enteritis (HE). This was the first and only time I encountered a "classical" HE outbreak that was associated with profuse bloody intestinal contents just as the textbooks describe. I didn't have the foresight to take photos. We immediately began an HE vaccination program and I never saw the classic lesions again. Obviously working with turkeys, enlarged marbled spleens associated with HE coupled with secondary *E. coli* infection are findings found almost on a daily basis, but the old textbook description of HE is a very rare occurrence nowadays.

In 1995 the meat turkey flocks in the Sanpete Valley became infected with a low path H7N3. From past experiences with AI we knew the infection would spread rapidly. The index flock exhibited no clinical signs. Only antibodies to prior infection were found by routine serologic testing at processing. However, the infection in subsequent flocks quickly host-adapted. Within a week or two of discovery, multiple flocks began showing respiratory signs. All major commercial meat turkey production in the State of Utah is raised within the Sanpete Valley – a mountain valley measuring approximately 12 miles wide by 25 miles long. It is not hard to imagine that the density of turkeys there was very high. State officials and the AVIC petitioned the USDA authorities for permission to use an autogenous killed H7 vaccine. After persistent negotiations, and the fact that the turkeys were isolated within a valley surrounded by mountains with no other turkey-producing entities within hundreds of miles, permission was granted. This was the first time a low path AI vaccine was allowed to be used in the US. The vaccine was manufactured by Maine Biological Laboratories with permission from Veterinary Services and Veterinary Biologics, USDA. Multiple vaccination crews were recruited, working under the direction of the MFC Veterinary Department. The widespread vaccination program was successful in reducing the continuation of the outbreak (See Proceedings of the Forty-Fifth Western Poultry Disease Conference, p. 32, 1996 for a summary).

Perhaps my most memorable case took place in a breeder flock of hens recently coming into production. This flock was brooded and grown in a new facility and geographic location where no turkeys had ever been raised. The

young hens were transferred to the lay facility and soon placed into the lay barn. About two weeks into production, the flock experienced a slight to moderate but noticeable drop in egg production. Extraneous management factors were systematically eliminated. As I was very diligent in regular serologic monitoring of our breeder flocks, I was able to closely investigate any untoward serologic response. Serologic findings two weeks after the production drop showed that titers for Newcastle, MG, PMV-3, *Bordetella avium*, and AI were within normal range; however, ELISA titers for HE were unusually high. On retrospective examination of serologic surveillance of this flock, it was discovered that the hens never seroconverted to HE. For some reason, the isolation during brooding and growout inhibited exposure to the virus. The conclusion was that the drop in production was attributable to exposure to HE during lay. I never saw this phenomenon again. Upon mentioning it to Yan, he said he has seen this before, but it is extremely rare because most turkeys become exposed to HE sometime in their lives (either naturally or vaccination) before egg production begins. Needless to say, a conscientious HE vaccination program was initiated for all of our subsequent breeder flock replacements – just in case. . . .

The assignment to move to the CUVDL and work not only with turkey producers but also to serve the table egg and commercial gamebird industries greatly expanded my professional horizons. Inherent with my extension assignment, I provided continuing education to commercial producer groups, backyard poultry hobbyists, and Extension agents

Perhaps one of my most satisfying extension opportunities was to learn about and work with the commercial gamebird industry. I was fortunate to assist in providing high level educational opportunities to gamebird producers in the Intermountain Area by collaborating with the Utah Gamebird Association in hosting a biennial Utah Gamebird Health and Management Symposium. Industry and academic experts were brought in from areas across the country to share their expertise with these regional producers.

One offshoot activity stemming from this relationship allowed me to collaborate with the Utah Department of Wildlife Resources on a project evaluating the blood and intestinal parasite load of wild chukars living in the west desert of Utah. “Coccidiosis” and “Chukars” are synonymous terms when these birds are raised in captivity. Interestingly, we saw no evidence of oocysts or coccidial infection in the wild birds. Low bird density coupled with hot dry desert conditions no doubt contributed to this finding.

I would be remiss in not acknowledging the principle people who have so greatly influenced me: My wife, Lisa, and our children who have been such a great support and encouragement; my good friends (some posthumous) Yan Ghazikhanian, Marcus Jensen, Royal Bagley, Art Bickford, Rich Chin, Gregg Cutler, Mark Bland, Lynn Bagley, and Dustan Clark; all other colleagues and friends whom I have met and worked with in various capacities within the WPDC and AAAP; the poultry and gamebird producers I have served; my colleagues at the CUVDL; and many others who, at the already serious risk of leaving someone out, am not able to adequately recognize within the confines of this brief paper.

My advice to young veterinarians:

1. Be willing to reach outside your comfort zone. There is always more to learn than just the bare minimum to hold down a job.
2. Forge relationships with other poultry professionals. Strive to become a “poultry scientist,” not merely a “poultry veterinarian.” If you do this, your opportunities for career development will be greatly expanded.
3. Balance your career with family and recreational activities. This will enhance your ability to focus and work hard when it is necessary.
4. Enjoy doing what you are doing! If you don’t, you’d better look for another way of making a living.
5. Remain curious throughout your career. Complacency is a killer.
6. Help others along the way. We all had many people help us – be appreciative of them.
7. And oh, by the way, always participate in the WPDC. . . .

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Underlined name denotes presenting author

COMPARATIVE ANALYSIS OF TYPING STRATEGIES FOR AVIAN REOVIRUS VARIANTS BASED ON PARTIAL S1 GENE SEQUENCE PHYLOGENY

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SUMMARY

Avian reovirus (ARV) is a non-enveloped double stranded RNA virus that causes disease in chickens leading to significant economic losses to the poultry industry. Due to its RNA nature and segmented genome, the virus is prone to mutations, reassortments and recombinations that result in the emergence of ARV variants. To date, many laboratories use dissimilar phylogenetic strategies to classify these variants reporting different genotypes (from 1 to 7) and some even sub-genotypes. In addition, different sequence sizes have been used by different laboratories. This study attempts to devise a common typing strategy for the classification of ARV variants through comparisons of different strategies used in two laboratories in the U.S and Netherlands.

INTRODUCTION

Avian reovirus (ARV) causes diverse health problems in broilers including arthritis and tenosynovitis (1,9). It is a non-enveloped virus with a double-stranded RNA genome that comprises of 10 segments. These segments are categorized into three groups based on their electrophoretic mobility: three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, and S4). Among these segments, S1 encodes the sigma C protein, a component of the viral capsid that plays a crucial role in host cell attachment and the generation of neutralizing antibodies (3,5,12). As a hypervariable protein, sigma C serves as a genetic marker for the classification of ARV strains into different genotypic clusters. Due to its RNA nature and segmented genome, the virus is susceptible to mutations, re-assortments, and genetic recombinations leading to the emergence of new ARV variants (4,11). To date, many typing strategies have been proposed to classify these variants (12). In 2003, Kant classified these viruses into five genotypic clusters (GCs) based on their amino acid homology of the partial S1 gene (7). In the United States, Lu used the same approach but classified ARV variants into six genotypic clusters in 2015 (10). Later, research groups in California and Georgia followed the same 6 GCs classification system until Sellers uncovered a 7th GC due to the high divergence of sigma C sequences in 2019 (4,12). However, like in other RNA virus classification systems e.g., IBV (6), a cut-off value needs to be defined easing the classification of variants based on amino acid identity. In this study, a common typing strategy was attempted to classify ARV strains. The aim was to establish an approach that could enhance the consistency and accuracy of ARV variants' classification across different laboratories. It involved thorough investigation of phylogeny using partial S1 gene sequences obtained by analyzing isolates from both laboratories with the goal of identifying similarities and divergences. The insights gained from this comparative analysis may contribute to the development of standardized protocols and facilitate more reliable ARV genotyping.

MATERIALS AND METHODS

Sampling and virus isolation. Tissues, including hearts, joints, intestines, and tendons, were collected from suspected cases of tenosynovitis in broiler chickens in California and the Netherlands. The isolation of avian reoviruses involved using chicken embryo liver (CEL) cells. Samples without detectable cytopathic effects (CPEs) within 5 days underwent a second passage (4). Subsequently, confirmatory reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to detect the conserved region of M1 gene in the ARV genome (13).

Molecular characterization. Partial S1 gene segments of 1088 bp and 802 bp were amplified and studied for the molecular characterization of ARV isolates. Primers used in this study for S1 gene segment amplification were obtained from Kant's publication (7). PCR products were purified using

QIAquick® PCR Purification Kit according to the manufacturer's protocol, and dsDNA concentration was determined using nanodrop. The purified PCR products were sent for Sanger sequencing. Obtained sequences were aligned with reference sequences along with three commercial vaccine sequences, S1133, 1733, and 2408 using Geneious Prime®. Phylogenetic analysis was performed by alignment of the 276 sequences, each comprising of 277 amino acids with MAFFT (2,8) and tree-building using the RAxML method with 1000 bootstrap replicates after trimming.

RESULTS

ARV isolation. Reoviruses were isolated from the samples particularly from tendons and intestines. No virus was isolated from hearts. Samples with positive RT-PCR for ARV were considered as cases.

Classification and molecular diversity. A phylogenetic tree of 277 amino acids was developed including isolates from both laboratories based on partial S1 gene (Figure 1). This figure compared the criteria used by both laboratories showing differences in the classification that denote lack of agreement of homology cut-offs.

DISCUSSION

This study involves sequences of ARV variants isolated from samples obtained between 2020 and 2022 along with reference sequences from GenBank. In this study, amino acid sequences of all isolates were trimmed and aligned to construct a phylogenetic tree. Genotypic clusters were assigned according to classification systems used in both laboratories i.e., a 7 GCs classification scheme used in the US and a 5 GCs classification scheme used in the Netherlands. Results showed that both schemes classify GC1, GC2 and GC5 identically. However, we could see that both GC3 and GC7 were included in the same GC3 in the Royal GD's classification. Similarly, isolates classified in GC4 and GC6 in the US system were depicted as GC4 in the Royal GD classification. This very simple exercise highlights the importance of standardizing classification schemes so they can be comparable between geographical regions. ARV isolates have a huge divergence both within and between genotypic clusters. Those differences should be investigated, and a common classification system should be adopted easing the comparison and further study of this pathogen's variability. This comparative analysis between methodologies from different laboratories may represent a crucial step towards standardizing ARV variant classification and it may be a first attempt towards a unified approach in ARV variant classification.

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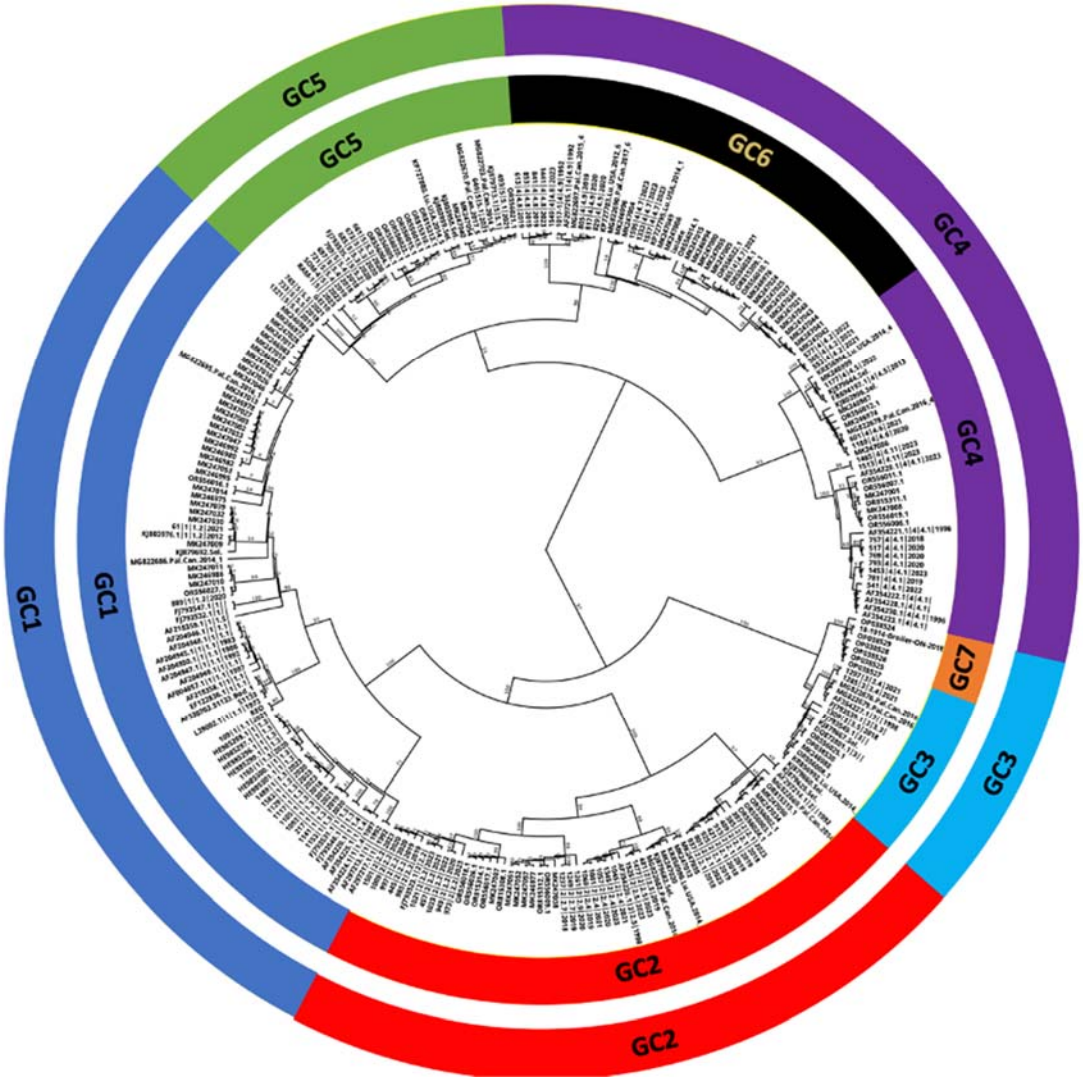
Figure 1. ARV phylogenetic tree of 277 amino acid sequences of the partial S1 gene, representing 276 ARV isolates, including vaccine strains. Constructed using MAFFT alignment and RAxML tools with 1000 bootstrap values. The inner circle displays the 7 Genotypic Clusters (7GCs) classification scheme in the United States, while the outer circle signifies the 5 Genotypic Clusters (5GCs) scheme in the Netherlands. Each color indicates a distinct genotypic cluster.



THE STATUS OF ANTIMICROBIAL USE AND RESISTANCE IN BROILER CHICKENS, TURKEYS AND LAYING HENS IN CANADA, 2022 UPDATES

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SUMMARY

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) monitors trends in antimicrobial use (AMU) and antimicrobial resistance (AMR) in sentinel broiler chicken, turkey and layer flocks. Between 2018 and 2022, total AMU, measured in number of defined daily doses per thousand bird-days at risk (nDDDvetCA/1,000 bird days at risk) decreased by 16% in broilers and 82% in turkeys. Only a limited number of layer flocks reportedly used antimicrobials; primarily bacitracin. *Salmonella* and *E. coli* isolates resistant to 3 or more classes of antimicrobials appear to be trending downwards in broiler chickens and turkeys. Notable results included nalidixic acid resistance (8%) in *Salmonella* from broiler chickens, driven by *S.* Enteritidis, and the occurrence of ciprofloxacin resistance in *Campylobacter* observed poultry industry wide (broiler chickens, turkeys, layers) suggestive of the widespread dissemination of quinolone resistance in bacterial populations in the poultry industry.

MATERIALS AND METHODS

In 2022, 94 broiler chicken, 71 turkey and 50 layer flocks were surveyed at the farm-level where AMU data and samples for bacterial culture/susceptibility testing were collected through a network of 16 poultry veterinarians and their producers. In brief, AMU data were summarized using routine CIPARS AMU measurements (count-based, weight-based and dose-based indicators) (1). For AMR, *Escherichia coli*, *Salmonella*, and *Campylobacter* were recovered from pooled fecal samples (four per flock) and isolates were susceptibility tested using the CMV5AGNF and CAMPY panel developed by the United States National Antimicrobial Resistance Monitoring System (2). AMR measurements include: % of isolates resistant to homologous antimicrobials such as ceftriaxone, gentamicin, and tetracycline, multidrug resistant isolates (for the purposes of our analysis, isolates resistant to ≥ 3 classes) and susceptible isolates. Detailed farm sampling, laboratory and analytic methods are described elsewhere (1). Five-year trends in AMU and AMR were assessed in relation to flock health information which were also collected through the farm questionnaires.

RESULTS AND DISCUSSIONS

Broiler chickens

AMU. Between 2018 and 2022, the percentage of broiler flocks [number of flocks exposed (n)/total flocks sampled (N)] exposed to medically-important antimicrobials (MIA) decreased from 77% to 71%. The nDDDvetCA/1,000 broiler chicken days at risk also decreased by 16%. Other AMU indicators such as mg/broiler chicken pre-harvest weight kg, referred to as mg/kg from this point forward, also decreased during the surveillance period. In 2022, the total AMU was 60 mg/kg broiler chicken biomass. Only five antimicrobial classes were reportedly used and the most frequently used classes were bacitracins (35 mg/kg), penicillins (10 mg/kg), and trimethoprim-sulfonamides combinations (8 mg/kg). A small proportion (1%) of World Health Organization's Highest Priority Critically Important Antimicrobials (WHO's HPCIA's) class (fluoroquinolones) was reportedly used to treat a flock that had high mortality.

AMR. Between 2018 and 2022, the percentage of multidrug resistant isolates decreased in *Salmonella* and *E. coli*, both by 11%. Multidrug resistant *Campylobacter* were detected only in 2019. Resistance to WHO's HPCIA's remained below 10% in 2022 in both *Salmonella* (ceftriaxone: 5%, nalidixic acid: 8%, ciprofloxacin: not detected) and *E. coli* (ceftriaxone: 2%, nalidixic acid: 5%, ciprofloxacin: <1%). Of the WHO's HPCIA, nalidixic acid resistance in *Salmonella* appears to be trending upward (3% in 2020, 8% in 2022), driven by *S.* Enteritidis. In *Campylobacter*, levels of resistance appear to be trending upwards for the quinolone antimicrobials. In 2022, a high-level of resistance to ciprofloxacin (34%)

was detected which exceeded the levels detected in 2020 (30%) and 2021 (22%).

Flock health. In 2022, the average percentage of mortality remained stable at 4%. Commonly occurring disease syndromes in broilers remained stable (yolk sac infections/septicemia, necrotic enteritis), however, miscellaneous bacterial diseases (combined salmonellosis, lameness due to Staphylococcus aureus and osteomyelitis) due to *Enterococcus cecorum* increased from 2% in 2021 to 8% in 2022.

Turkeys

AMU. Between 2018 and 2022, the percentage of turkeys exposed to MIA decreased from 63% to 14%. This decrease corresponded with a substantive drop (82%) in the nDDDvetCA/1,000 turkey days at risk. A drop was also observed in other AMU indicators measured by CIPARS (mg/kg turkey pre-harvest weights) during the surveillance period. In 2022, total use was 9 mg/kg turkey biomass. Only 6 antimicrobial classes were reportedly used and the most frequently used classes were penicillins (4 mg/kg), orthosomycins (3 mg/kg), and bacitracins (2 mg/kg). The ranking of antimicrobials used in turkeys differed slightly compared to the broiler chickens. Less than 1% of the total AMU was attributed to the fluoroquinolone class of antimicrobials which were used to treat a flock that had high mortality.

AMR. Between 2018 and 2022, the percentage of multidrug resistant isolates decreased in *Salmonella* and *E. coli* by 29% and 20%, respectively. Multidrug resistant *Campylobacter* were detected since 2021 but have been sustained below 5%. Resistance to WHO's HPCIA's were detected at low levels in 2022: 3% ceftriaxone resistance in *Salmonella*, 3% in ciprofloxacin and 3% nalidixic acid in *Salmonella,* and 2% nalidixic acid and 2% ciprofloxacin in *E. coli*. In *Campylobacter*, resistance to ciprofloxacin (11%) dropped from its 2021 level (19%).

Flock health. In 2022, the average percentage of mortality slightly increased compared to 2021 (6% to 6.6%). Commonly occurring disease syndromes in turkeys (airsacculitis, yolksacculitis and septicemia) were stable (3% to 6%).

Layers

AMU. Only bacitracin (n = 10 flocks) was reportedly used in 2022 for treating necrotic enteritis.

AMR. Resistance to 3 or more antimicrobial classes was observed only in *E. coli* isolates at very low levels (1%). More than half of the isolates (*E. coli*, *Salmonella*, and *Campylobacter*) were susceptible to the panel of antimicrobials tested. The AMR profiles seen in layers varied from that of broiler chickens and turkeys.

Flock health. Overall, necrotic enteritis and coccidiosis were occasionally reported but no other syndromes affecting mortality or egg production were reported.

CONCLUSION

Ongoing surveillance for AMU at the farm level indicated that quantity of antimicrobials decreased between 2018 and 2022 and fewer antimicrobial classes were being used for prevention of these diseases. Resistance levels appear to be stable across the species under surveillance, however, the occurrence of antimicrobials, deemed as WHO's HPCIAs needs to be monitored closely.

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Figure 1. Distribution of antimicrobials used in broiler chickens in 2022, mg/kg broiler chicken biomass. Chart excludes flavophospholipids, a non-medically important antimicrobial (1%).

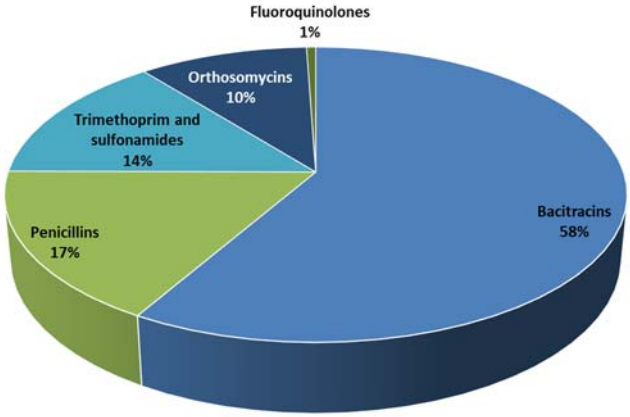
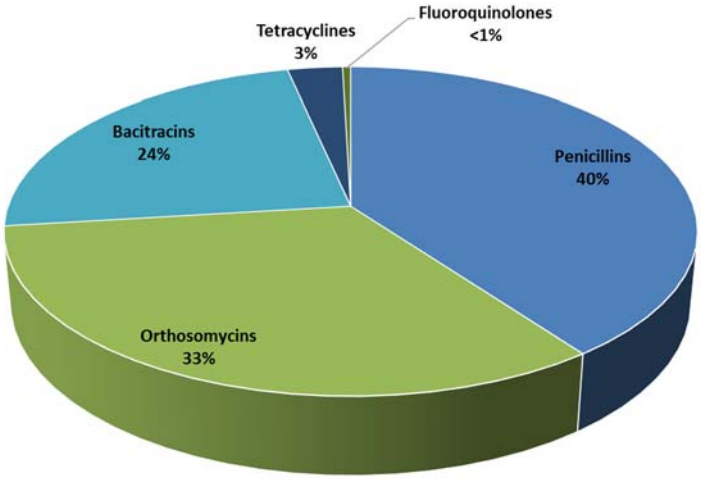


Figure 2. Distribution of antimicrobials used in turkeys in 2022, mg/kg turkey biomass. Chart excludes flavophospholipids, a non-medically important antimicrobial (13% of total use).



SPOTTY LIVER DISEASE CONTROL IN CAGE FREE LAYERS: FIELD EXPERIENCE WITH AN AUTOGENOUS CAMPYLOBACTER HEPATICUS VACCINE IN THE USA

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INTRODUCTION

Spotty liver disease (SLD) is an acute infectious disease of layer chickens characterized by multifocal necrotic lesions in the liver, increased mortality, and drops in egg production. Although the disease has been recognized since the 1950's, its etiological agent Campylobacter hepaticus was not identified until recently. The disease is becoming more prevalent in Europe and Australia, especially in cage-free layers. SLD caused by *C. hepaticus* was first reported in the United States by Gregory *et al*. in *Avian Diseases* in 2018. Since then, many layer operations in the USA have experienced economic losses due to SLD.

BACKGROUND

Cage-free layer flocks producing antibiotic-free eggs in Central USA started experiencing SLD breaks of increasing severity in 2019-2020. SLD tended to repeat in affected farms, occurred at earlier ages with higher mortality and more severe drops in egg production. Due to the limited therapeutic alternatives and difficulties controlling SLD in flocks producing antibiotic-free eggs, *C. hepaticus* vaccination was introduced to control SLD specifically in cage-free, free-range, and pasture raised flocks.

OBJECTIVE

The objective of this study was to evaluate the effect of vaccination with an autogenous killed vaccine to control of *C. hepaticus* in laying hens in the USA.

MATERIAL AND METHODS

Bile and liver samples were collected from 16 flocks of brown laying hens experiencing SLD-like lesions between 26 and 67 weeks of age from October 2020 to November 2021. Campylobacter hepaticus was isolated from 52/127 samples (41%) and its presence was confirmed by qPCR. Whole genome sequencing was performed on 20 of these isolates using Illumina MiSeq. Raw sequence data for each isolate was processed using SPAdes version 3.15.3. Sequences were normalized and error corrected prior to Denovo assembly. Denovo assemblies were analyzed using Geneious Prime software version 2021.2.2 and ABRICATE to search the virulence factor database (VFDB) at the National Center for Biotechnology Information (NCBI), and antibiotic resistance genes using MEGARES (Figure 3). Based on this information, two of the October 2020 isolates were selected to produce an autogenous vaccine according to the Code of Federal Regulations for Autogenous Biologics (9CFR 113.113). A total of 4,595,000 doses of Autogenous vaccine were manufactured using ENABL® adjuvant. Vaccination started in April 2021. The vaccine was applied by the intramuscular route in the breast following two different programs: A) One dose at 12 weeks of age or B) two doses at 8 and 12 weeks of age when other vaccines were also applied (inactivated NDV/IBV/*Salmonella* Enteritidis, avian encephalomyelitis, fowl pox, and cholera).

RESULTS

No serious adverse effects were reported after vaccination with this *Campylobacter hepaticus* bacterin. Farms in which SLD had been confirmed by *C. hepaticus* culture and/or PCR were monitored after vaccination (n=13), and flock performance was compared. Performance curves of the most severely affected farms before and after vaccination are shown in Figures 1-4. Figure 5 shows the difference in egg production between vaccinated flocks and SLD+ flocks by farm at similar ages. Improvements in hen mortality and egg production ranging from 11 to 51 eggs/hen were observed in 10/13 farms (77%). In farms not showing an improvement after vaccination, *C. hepaticus* isolates differed genetically from the ones included in the autogenous vaccine (Codes LS21-4363 and LS21-4365 >1200 SNPs difference, absence of rfbC gene, and presence of waaF gene, and presence of TETO gene coding for tetracycline resistance ribosomal protection proteins in LS21-4365). Those

isolates should be taking in consideration when formulating future autogenous vaccine serials for broader protection.

CONCLUSION

Overall, reduced incidence and severity of SLD was observed in C. hepaticus-vaccinated flocks. This resulted in improved livability and egg production, making autogenous C. hepaticus vaccination a cost-effective tool for the producer to control SLD.

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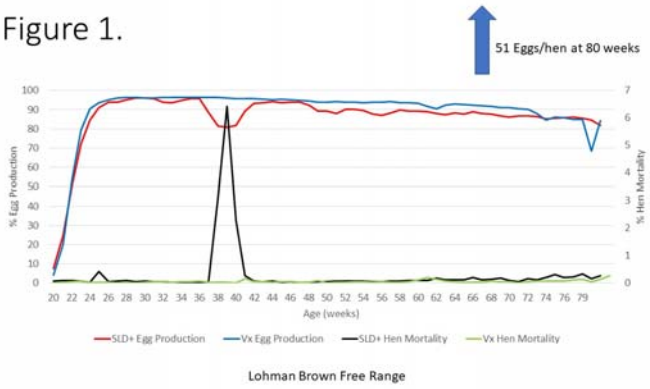
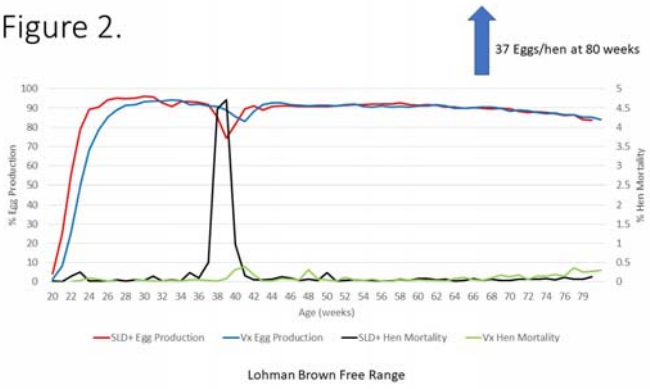
Figure 1.



Lohman Brown Free Range

Figure 2.



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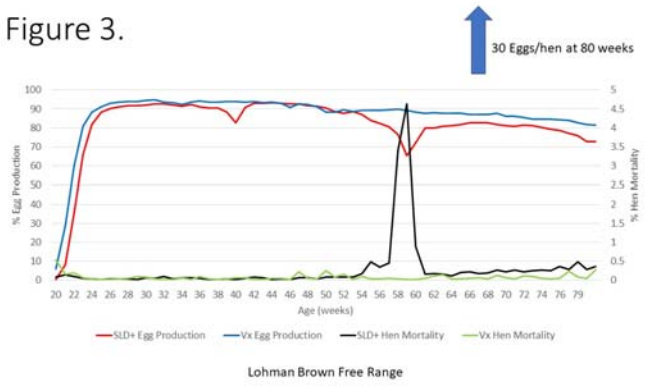

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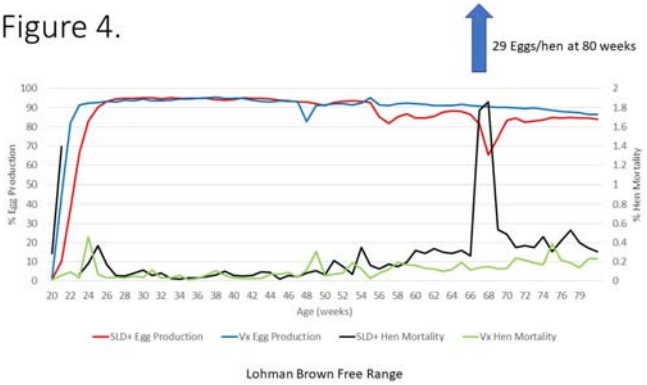

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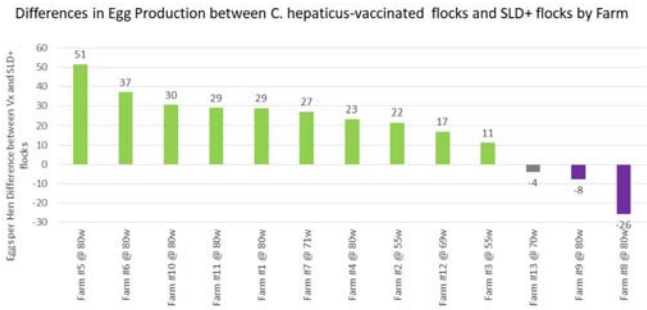

DETECTION OF HVT-ILT VACCINES CARRYING THE GLYCOPROTEIN I GENE OF INFECTIOUS LARYNGOTRACHEITIS VIRUS IN VACCINATED BIRDS

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SUMMARY

We present the development of a real time PCR (rt-PCR) for the detection of recombinant HV-ILT vaccines (rHVT) carrying the glycoproteins I and D of infectious laryngotracheitis virus in feather pulps of vaccinated birds. The glycoprotein I specific rt-PCR (gI rt-PCR) was compared to a highly specific and sensitive HVT rt-PCR that amplifies a segment of the unique ORF1 gene (ORF1 rt-PCR). Feather follicle samples collected at 7-, 14- and 21-days after *in ovo* vaccination were evaluated with both, the gI rt-PCR and ORF1 rt-PCR assays. Average gI rt-PCR CT values of 30.3, 24.4 and 28.5 were detected at 7-,14- and 21- days of age, respectively. High correlations (≥ 0.86) were observed between the CT values obtained with the gI rt-PCR and ORF1 rt-PCR assays. The field applications of the developed gI rt-PCR test include the confirmation of in ovo and subcutaneous administration of rHVT-ILT vaccines carrying the gI gene and the evaluation of hatchery vaccination techniques.

INTRODUCTION

ILTV or Gallid herpesvirus 1 is an alphaherpesvirus that causes a respiratory disease in chickens and significant economic losses to the poultry industry worldwide (Maekawa *et al*., 2019; Bagust *et al*., 2000; Davison *et al*., 2009). Severe forms of the disease are characterized by gasping, expectoration of bloody mucus and moderate to high mortality by asphyxia associated with the presence of tracheal plugs (Guy *et al*., 2008). Control of the disease is based on strict biosecurity and the implementation of vaccination programs in affected areas (Guy *et al*., 2008; García *et al*., 2019).

Live commercial vaccines have been successfully used to control outbreaks. However, their capacity to spread from bird to bird (particularly the chicken embryo origin vaccines), regaining virulence and the establishment of latent infections with sporadic reactivation leading to virus shedding are a matter of concern (Hughes *et al*., 1987; Menendez *et al*., 2014; Guy *et al*., 1990; Guy *et al*., 1991). As a response to the frequent ILT epizootics related to CEO vaccines, a new generation of recombinant vaccines using fowl poxvirus and herpesvirus of turkey (rHVT) as vectors were developed (Maekawa *et al*., 2019b). rHVT-ILT vaccines are characterized by persistent viremia and the ability of the HVT vector to replicate in lymphocytes in a highly cell-associated manner, establishing long lasting cell mediated immunity (Heller *et al*., 1987; Gimeno *et al*., 2011; Esaki *et al*., 2013). ILTV envelope glycoproteins expressed in commercial HVT vectors play major functions in herpesvirus infection and replication (Devlin *et al*., 2006; Basavarajappa *et al*., 2014):

- Glycoprotein I forms heterodimers with glycoprotein E favoring cell-to-cell virus spread while avoiding host immune defenses.
- Glycoprotein B is essential for infectivity (membrane fusion and virus penetration).
- Glycoprotein D binds to target host cell receptors and has a superior envelope incorporation and cell surface expression leading to induction of a superior protective immune response than glycoprotein B.

The objective of this study was to develop and validate a rt-PCR assay for the detection of the glycoprotein I gene present in two commercial rHVT-ILT vaccines (rHVT-ILT and rHVT-ND-ILT). The sensitivity and reproducibility of the developed gI rt - PCR assay was compared with a previously developed rt-PCR targeting the HVT ORF 1 (ORF1 rt-PCR).

MATERIALS AND METHODS

Birds and vaccines. Broiler eggs were divided in three groups, a control group and two groups vaccinated *in ovo* with the rHVT-ILT or the rHVT-ND-ILT commercial vaccines expressing the glycoproteins I and D of infectious laryngotracheitis virus. At hatching, birds from the control and vaccinated groups were tagged and maintained in

separate isolation units until the end of the study. Primary feather samples collected from the wings of each bird at 7-, 14- and 21- days of age were collected in lysing matrix D tubes and storage at -80 C.

DNA extraction. Feather pulps were collected in lysing matrix D tubes (MP Biomedicals, Santa Ana, CA) containing 1.0 mL of phosphate-buffered saline (PBS) with 2% antibiotic-antimycotic (Invitrogen) and 2% newborn calf serum (NBCS; Gibco, Waltham, MA). Samples were homogenized in the FastPrep-24™ 5G instrument (MP Biomedicals) before storage at −80 C. DNA extraction was performed using the MagaZorb® DNA extraction mini-prep kit (Promega, Madison, WI) following the manufacturer's recommendations.

Glycoprotein I PCR (gI rt-PCR). PCR primers an probe were designed based on a highly conserved region of the gI gene of infectious laryngotracheitis virus. The PCR reaction consisted of three thermocycle stages, stage 1 at 50°C for 120 seconds, stage 2 at 95°C for six hundred seconds and stage 3 with forty cycles of 95°C for 15 seconds with optics off and 60°C for 60 seconds with optics on.

HVT PCR (ORF1 rt-PCR). HVT genome viral load in control and vaccinated groups was assessed using the same feather pulp samples evaluated with the gI rt-PCR assay. HVT genome was detected using a previously developed rt PCR that amplifies a segment of the unique ORF1 gene of HVT. This real time PCR assay has proven to be highly specific and sensitive for the detection of the genome of recombinant and conventional HVT vaccines in chickens (Islam *et al*., 2004).

RESULTS

The presence of the gI gene in feather follicle samples from birds vaccinated with the rHVT-ILT was detected in 90%, 88% and 100% of the vaccinated birds at 7-, 14- and 21-days post-vaccination, respectively. Detection of the ORF1 gene was observed in 70%, 100% and 89% of the vaccinated birds at 7-, 14- and 21- days of age, respectively. Correlations between Ct values obtained by the gI rt-PCR and ORF1 rt-PCR assays are presented in Figure 1. Correlation coefficients of 0.93, 0.88 and 0.98 were observed at 7, 14 and 21 days of age, respectively. Furthermore, the overall correlation between the two real-time PCR assays was high (r= 0.91).

In rHVT-ND-ILT vaccinated birds, the presence of the gI gene in feather follicle samples was detected in 100%, 94% and 100% of the vaccinated birds at 7-, 14- and 21-days of age, respectively. Detection of the ORF1 gene was observed in 90%, 89% and 100% at 7-, 14- and 21- days of age, respectively. Correlations between Ct values obtained by the gI rt-PCR and ORF1 rt-PCR assays are presented in Figure 2. Correlation coefficients of 0.96, 0.84 and 0.79 were observed at 7-, 14- and 21- days of age, respectively. Furthermore, the overall correlation between the two real-time PCR assays was high (r= 0.85).

CONCLUSION

The gI rt-PCR assay evaluated in this study was able to detect the presence of the rHVT-ILT and rHVT-ND-ILT vaccines expressing the glycoprotein I in feather follicles of birds vaccinated with two rHVT-ILT vaccines expressing the glycoprotein I and D of infectious laryngotracheitis virus. The developed assay is a practical and reliable tool to confirm the hatchery administration of rHVT-ILT vaccines carrying the glycoprotein I and to evaluate vaccination techniques.

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Table 1. Reagents, sources, stock concentrations and volume of probes and primers designed to amplify the glycoprotein I gene present in the rHVT-ILT and rHVT-ND-ILT vaccines expressing the glycoproteins I and D of infectious laryngotracheitis virus.

Reagents	**Sources**	**Stock Concentration**	**Concentration / Rx**	**Volume/Rx (ul)**
Tagman Universal Mix II x2	Thermo Fisher	1X		12.5
dH2O	x	x	x	x
Primer Coll F	IDT	10uM	.5uM	1.25
Primer Coll R	IDT	10uM	.5uM	1.25
Primer gI F	IDT	10uM	.5uM	1.25
Primer gI R	IDT	10uM	.5uM	1.25
Probe Coll	ABI	2uM	.1uM	1.25
Probe gI	ABI	2uM	.1uM	1.25
Template				5
Total				25

Figure 1. Correlation coefficients between Ct-values obtained by the gI rt-PCR and ORF1 rt-PCR assays in feather follicles of rHVT-ILT vaccinated birds at 7-, 14- and 21-days post-vaccination.

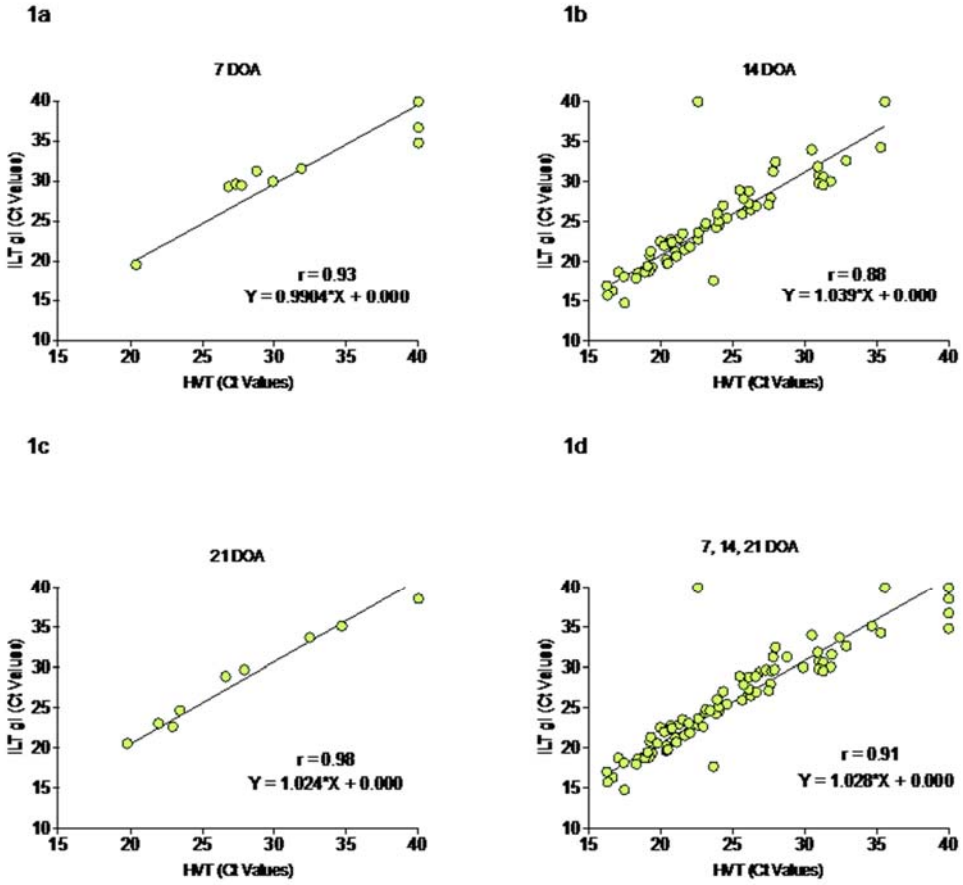
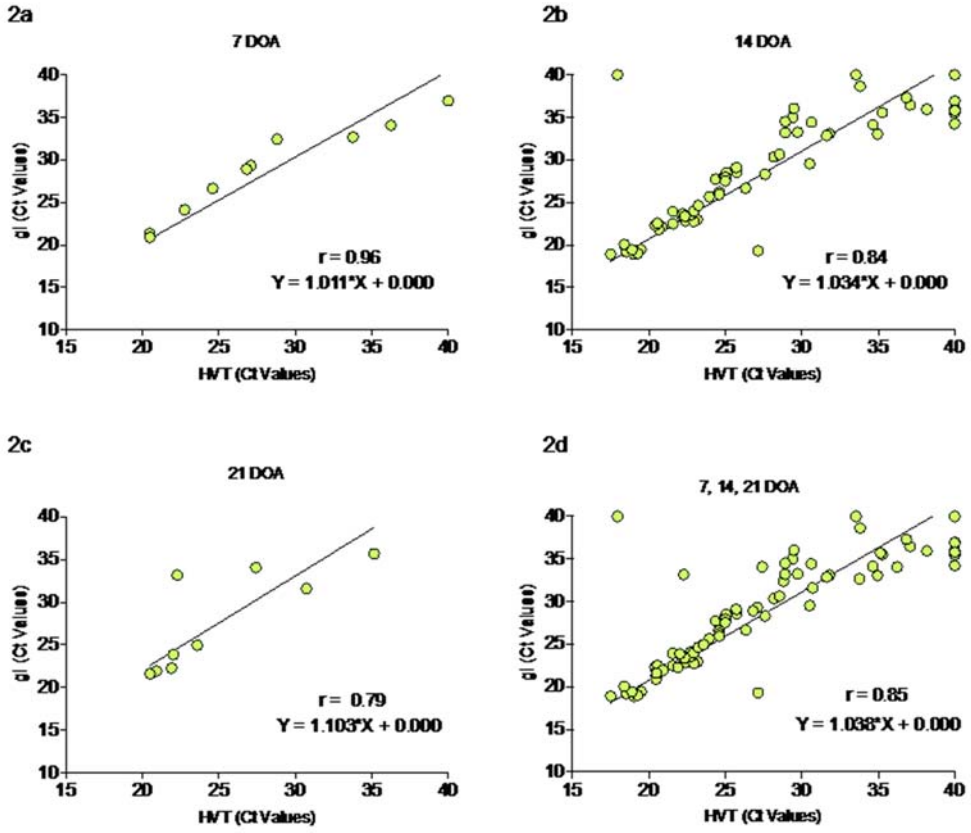


Figure 2. Correlation coefficients between Ct-values obtained by the gI rt-PCR and ORF1 rt-PCR assays in feather follicles of rHVT-ND-ILT vaccinated birds at 7-, 14- and 21-days post-vaccination.



DIFFERENTIATION OF NON-PATHOGENIC FROM PATHOGENIC *ENTEROCOCCUS CECORUM* FIELD ISOLATES BASED ON THE DETECTION OF THE CPSO GENE AND ITS CORRELATION WITH MACROSCOPIC LESIONS

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SUMMARY

Clinical presentation of *Enterococcus cecorum* (EC)-associated disease in broiler chickens is characterized by signs related to septicemia, lameness, and sometimes paralysis (chickens older than 4 weeks). During the past few years, the incidence of this disease has increased worldwide. The *cpsO* gene was recently reported to successfully differentiate between pathogenic and commensal EC strains. The objective of this study was to differentiate between commensal and pathogenic EC isolates recovered from field cases. *Enterococcus* spp. isolates (*n*=348) recovered from cases submitted to Mississippi State University's Poultry Research and Diagnostic Laboratory (PRDL) during 2023 were analyzed. Isolates originated from broiler breeders, layer breeders, hatcheries, and broiler chickens. Selected EC isolates (*n*=100) were analyzed phenotypically and genotypically to differentiate pathogenic and commensal strains. EC was recovered from samples of the heart, joints, ovary, testicles, midgut, and ceca. The *cpsO* gene was detected only in two breeders isolates from the heart and hock joint. In contrast, EC was frequently isolated from broilers, mainly from the heart and femoral head lesions; with the *cpsO* gene detected in a high percentage (65%). The presence of this gene was highly correlated with EC isolated from lesions observed in clinical disease cases, suggesting the pathogenic nature of these EC isolates. EC was not isolated from any hatchery samples.

INTRODUCTION

Enterococcus cecorum (EC) is an emerging cause of disease and significant economic losses in the United States poultry industry, especially in the broiler sector (1,2,3). The EC-related disease has implications for both the health of the birds and their productive performance, and it is also considered of great importance in terms of animal welfare, especially due to the lameness problem observed in chickens affected by arthritis and femoral head necrosis (FHN) (1). EC has been associated with a marked increase in mortality, processing plant condemnations, and poor feed conversion worldwide (4). Additionally, this bacterium is showing high levels of antibiotic resistance, which has generated alarm in terms of public health due to the potential presence of resistant EC in chicken meat (1,4).

The number of cases of broiler chickens diagnosed with EC-related disease at the PRDL has been exponentially increasing over the past four years. In acute disease, clinical signs are not commonly observed. However, in some cases clinical presentation is related to the site of infection and includes lameness, depression, decreased feed intake, and consequently weight gain (5). There are no specific data available on morbidity and mortality caused by EC disease. However, some authors report that EC-related morbidity can be as high as 35 %, and mortality up to 15% (3,4). The main gross lesions include pericarditis, perihepatitis, FHN, and vertebral osteoarthritis (VOA)/osteomyelitis (1,6).

EC is a commensal microorganism in the chicken gastrointestinal tract (GIT) which may have acquired characteristics that enable it to translocate, invading internal organs such as the heart and liver, and sometimes the vertebra, causing systemic disease in broilers. Despite recent advances in understanding the genetic basis for increased virulence in pathogenic EC, little is known about the critical steps in its pathogenesis (3). The source of pathogenic EC is still unknown. Pathogenic strains of EC have been identified over the past 20 years, and genetic analyses have demonstrated that these strains are genetically related and share several putative virulence genes (4). In 2015, Borst *et al*. identified a variable 19 gene region using whole genome sequencing and postulated that the *cpsO*gene related to the capsule could be used to differentiate between pathogenic and non-pathogenic strains. In 2023, Walker *et al.* (7) developed a protocol based on the detection of the *cpsO* gene that facilitated the detection of pathogenic EC from different types of samples, including EC isolated from the vertebra, heart, and air sacs.

The objectives of this study were 1) to evaluate the incidence of gross lesions caused by EC in chickens; 2) to correlate the site of EC isolation with the presence of the *cpsO* gene; and 3) to compare the recovery of EC and the presence of the *cpsO* gene from extraintestinal organs with that from intestinal samples.

MATERIALS AND METHODS

Isolates collection. During 2023, a total of 348 *Enterococcus* spp. isolates were recovered from cases submitted to the PRDL. Cases had a history of increased mortality and signs of infection or lameness. The submissions included chickens for necropsy, and samples of organs collected from affected birds. The following information was collected from each case: Bird type and age, clinical signs, mortality, gross lesions at necropsy, and sites of isolation. One hundred EC isolates were randomly selected for further analysis.

Bacterial culture and EC identification. Samples submitted directly to the PRDL and collected at necropsy were cultured following the established PRDL protocol. Briefly, each sample was streaked onto Columbia Nalidixic Acid (CNA) agar plates (Remel Thermo Fisher Scientificä, Waltham, MA) and incubated under microaerophilic conditions for 24 hrs. Isolate identification was performed by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) using a Vitek® MS instrument (bioMerieux, Inc, Durham, NC).

DNA extraction and PCR protocol. To perform DNA extraction, one pure bacterial colony from blood agar was transferred into brain heart infusion broth (BHI) (Thermo Scientific™ Remel) and incubated overnight at 37°C under microaerophilic conditions. DNA Extraction was performed following the manufacturer's instructions on 1000 µL of the incubated broth using MagMax™ Pathogen RNA/DNA kit.

PCR amplification was performed using primers *CpsO*-F GCGATTGTTCCAAAGGTGTTAG and *CpsO*-R AGTTTGAATGGCAAAGCTAATTC (Eurofins) (7). The master mix was prepared following PRDL protocols as follows: Three microliters of DNA were added into a mix of 1 µL of reverse and forward primers, 20 µL of water, and 25 µL of GoTaqÒ Hot Start Green Master Mix M5122 (Promega Corporation, Madison, WI) to obtain a final volume of 50 µL. PCR protocol developed by Walker *et al*., 2023 was modified to be carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany), including the following steps: 94°C for 4 min, 30 cycles of 95°C for 1 min, 30 cycles of 50°C for 1 min, 30 cycles of 72°C for 1 min, and final extension of 72°C for 7 min.

Pulse gel electrophoresis (PGEF). Amplified PCR products were resolved using QIAxcelÒ capillary electrophoresis system toidentify the presence of a band with the expected nucleotide size of approximately 195 bp amplicon. Non-pathogenic *Enterococcus cecorum* ATCC (43198Ô) and a wild type of *Escherichia coli* isolated in the PRDL were used as reaction-negative controls. Water was used as the Master Mix negative control. Two EC isolates previously characterized by the PRDL as pathogenic following the Walker *et al*., 2023 protocol (*cpsO* gene PCR positive) were included as positive controls.

Statistical analysis. Descriptive statistics were performed to measure the central tendency and variability of the data obtained from the 100 EC cases selected for this study. Incidence of gross lesions and *cpsO* PRC detection were analyzed by chi-square test. An alpha level of 0.05 was used for statistical significance.

RESULTS

Type of birds and age. EC was recovered from broiler chickens (n=71), broiler breeders (n=21), broiler breeder pullets (n=6), and commercial layer breeders (n=2. One from 31-weeks old breeders, and one from 7-day-old pullets). In broilers, 55% of the EC recovery came from chickens between 3 and 4 weeks of age (n=39), whereas, in the other bird types, EC was isolated from birds ranging between 4 to 61 weeks of age (n=28).

Clinical signs and mortality. Lameness was the most representative clinical sign reported among all necropsy cases (75%); followed by birds reluctant to move (27%). Additional clinical signs include poor uniformity (19%), and depression (8.3%). Before the birds' submission, daily mortality averaged 0.3%.
A sudden increase of up to 0.8% daily mortality was reported in 13% of the cases, being virulent EC isolated from the heart (92%), and from the heart as well as femoral head or hock joints (77%).

Gross lesions. Out of the 100 EC cases selected, gross lesions identified were pericarditis (58%), perihepatitis (16%), FHN (47%), arthritis (31%), and vertebral osteomyelitis/VOA (9%). Differences between gross lesions found in broilers and broiler breeders/pullets are illustrated in Figure 1.

Site of isolation. EC isolation sites included septicemic lesions observed in the heart and liver (60%), and skeletal lesions such as those observed in the femoral head, hock joint, and vertebra (12%). 15% of isolates were recovered from the gastrointestinal tract. EC was not recovered from any of the hatchery samples (n=52). These samples included embryo

mortality (n=38), egg yolk and egg yolk swabs (n=15), and environmental swabs (n=12).

Gene detection. Figure 2 shows significant differences ($P < 0.05$) in cases exhibiting pericarditis, perihepatitis, FHN, and arthritis, where the *cpsO* gene was detected. In contrast, no significant difference ($P > 0.05$) was observed in VOA cases. None of the EC samples from the gastrointestinal tract were PCR-positive for the *cpsO* gene. Additionally, this gene was not present in any EC isolated from birds younger than three weeks of age.

DISCUSSION

Historically, EC has been associated with subclinical septicemia followed by a skeletal phase referred to as enterococcal spondylitis (3). This septicemia in commercial chickens characterized by pericarditis, perihepatitis, and femoral head necrosis/osteomyelitis could be mainly observed during the second and third weeks of life (1,6), whereas skeletal presentation commonly starts after the fourth week of life (1,3,5). In our study, 55% of the EC cases came from birds within three and four weeks of age, which aligns with the literature and demonstrates age susceptibility. Broiler chickens are particularly prone to EC infection compared to other poultry types (1,10). In our study, there was a significant difference ($P<0.05$) in the incidence of EC-associated septicemic lesions (pericarditis and perihepatitis), and skeletal lesions (FHN, arthritis, and spondylitis) in broilers, when compared to broiler breeders. Additionally, EC was not isolated from any hatchery sample. Interestingly, EC was isolated in commercial layer breeders (31-weeks old) from bone marrow and liver, and in layer pullets from heart and yolk sac (8 days-old) with a report of a high first week mortality). The *cpsO* gene was not detected in the 31-week-old breeder sample. However, this result suggests investigating the presence of EC in commercial layers and evaluating the potential impact of EC in commercial layers.

Differentiating between commensal and pathogenic EC strains *in vitro* may include the utilization of a mannitol fermentation test (9), assessing antibiogram profiles (4), and detection of virulence genes by PCR (7,9). The *cpsO* gene was recently reported to successfully differentiate between pathogenic and commensal EC strains. This gene was evaluated in the current study. Our results indicate that the presence of this gene is highly correlated ($P < 0.05$) with an increased incidence of pericarditis, perihepatitis, FHN, and arthritis. There was not a significant difference ($P> 0.05$) in the c*psO* detection from all VOA lesions (Figure 2). However, a higher number of VOA EC isolates is required to effectively evaluate this lesion and the presence of the virulent gene.

EC strains are usually considered pathogenic when they are recovered from extraintestinal organs, whereas commensal strains are generally isolated from the intestines (9,11). Intestinal samples (n=14) where EC was isolated were evaluated for c*psO* gene presence. c*psO* gene was not detected in any sample, indicating their commensal characteristic.

The results of this study confirm that the detection of the *cpsO* gene can be used for the identification of pathogenic EC isolates from organ samples presenting classic lesions of systemic EC disease in birds older than two weeks, and to differentiate them from EC recovered from younger birds or from intestinal samples where *cpsO* was not present. This tool will be very useful for the effective discrimination of pathogenic and commensal EC, contributing to the accurate diagnosis of further control of EC-associated disease.

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

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Figure 1. Comparison of gross lesions in broilers and broiler breeder/pullets. Chi-square was used to determine significance at $P < 0.05$. A,B indicates significant differences between gross lesions and type of bird.

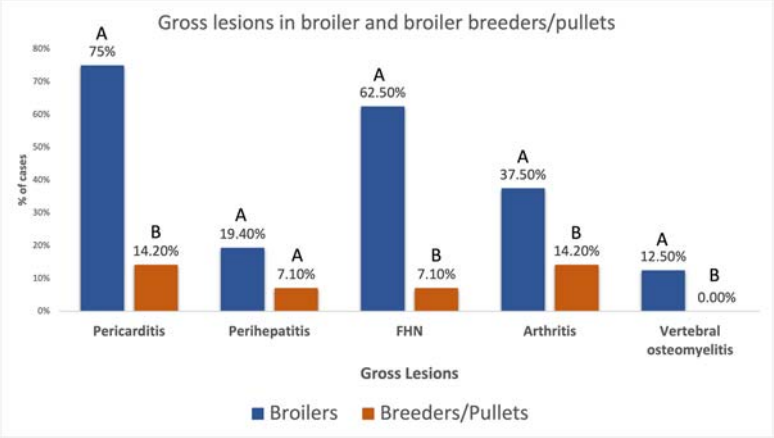
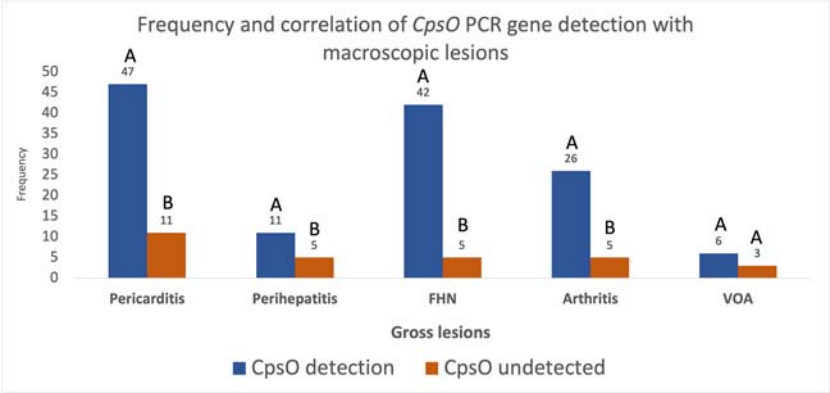


Figure 2: *CpsO* gene PCR detection and its correlation with macroscopic lesions. Chi-square was used to determine significance at $P < 0.05$. A, B indicates significant differences between gross lesions and *cpsO* detection.



INVESTIGATING THE EFFICACY OF A NOVEL VACCINE AGAINST *CAMPYLOBACTER HEPATICUS* USING A CHALLENGE MODEL THAT INCLUDES MULTIPLE STRAINS OF *C. HEPATICUS* FROM FIVE DIFFERENT STATES IN THE US

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Spotty liver disease (SLD) caused by *C. hepaticus* is an important cause of disease in layers. Currently, there are no approved treatments, or vaccines available. This study assessed the transmission of a novel live *C. hepaticus* vaccine to sentinel layers and the efficacy of the vaccine in a challenge model. Thirty-four commercial brown hens (16 wks) were assigned to two groups and received the live vaccine strain. Five sentinel birds were added to each group after vaccination to evaluate transmission of vaccine to naïve non-vaccinated chickens. At 18 weeks of age, one group received a booster of an inactivated vaccine. A non-vaccinated, challenge group (n= 17) received an oral placebo. All groups were orally challenged using a 5-strain cocktail of *C. hepaticus* (1x1011 cfu/mL) at 24 weeks of age. After challenge, five sentinel birds were added to each group. On days 8, 15, and 16 post-challenge, a sub-population of vaccinated, sentinels, and challenged hens were euthanized and tissues collected for bacterial culture, histopathology and lesions scored. Data generated provides insight on vaccines to control *C. hepaticus* in layer hens.

INTRODUCTION

Spotty liver disease (SLD) has emerged as an important cause of disease in table egg layers and layer breeders in the United Kingdom (UK), Australia, Jordan, and in the United States (US). In the US it has been detected in the midwestern and southern states (1,2,4,6,12); The organism implicated in SLD, *Campylobacter hepaticus*, has been reported as the causative organism resulting in multifocal lesions on the livers of infected birds which has resulted in reduced egg production, reduced egg size and increased mortality of highly valuable hens. Recently, *Campylobacter billis* has also been isolated from chickens with spotty liver disease and has been suggested as a second *Campylobacter* species causing SLD in poultry (8). *C. hepaticus* is a Gram-negative organism that grows under microaerophilic conditions at 37-42°C, has an S-shaped cell morphology with a single bipolar flagella. The colony morphology is cream-colored, flat-spreading, and has a wet appearance (11). The disease appears to affect hens around peak production (26 to 30 weeks of age), but it has been reported in birds as young as 25-26 weeks of age (9). Of most significance, is the emergence of *C. hepaticus* in birds that are housed in free-range sheds, though reports in the midwestern and southern US also documented morbidity due to *C. hepaticus* in caged layers (4). In one study, the strains of *C. hepaticus* implicated in disease in the US appear to be highly similar strains implicated in disease in the UK and Australia, suggesting the emergence of a new pathogen affecting the world's egg laying hens (4). Currently, mortality rates as a result of *C. hepaticus* are relatively unknown, however, based on studies from the UK and Australia, weekly mortality rates as high as 1-4% have been reported with total mortality as high as 10% (5,7). Decreased egg production is a significant concern with losses in the range of 10-25% being reported (3). Necropsy of diseased birds show characteristic multifocal spots on the liver of 1-2 mm in diameter, fibrinous perihepatitis, and splenomegaly with mottling (10). This project is one of the first to approach development of a vaccine candidate to assess the potential protection and control measures for *C. hepaticus* in challenged birds. Results will based on clinical signs, gross lesions scores of the liver, mortality, and transmission of *C. hepaticus* to naïve exposed chickens. The organism's presence will be determined using bacteriology, PCR, and histopathologic scores. A potential outcome of this project is a novel vaccine and designed vaccine regime that will eliminate *C. hepaticus* that is threatening the health and welfare of layer and poultry production in the US.

MATERIALS AND METHODS

Development of *C. hepaticus* vaccine in the lab. Here, our lab will build on some preliminary data we have already generated. One of the challenges on developing a vaccine for *C. hepaticus* is the limited data available on the pathophysiology of *C. hepaticus*. It takes about seven days for *C. hepaticus* to grow on

blood agar media, therefore growth of large quantities of the organism has been a challenge for some companies. Our lab will grow and produce a live and killed vaccine using enrichment and culture protocols developed. We will run protocols to ensure the live vaccine is stable and live before use in dosing birds. One of our sequenced and well characterized strains will be selected as the candidate vaccine strain. Ten microliters of the frozen stock solutions will be picked and plated directly on blood agar with incubation of the plates and broth at 37°C and 42°C for up to 7d under microaerophilic conditions using Mitsubishi Anaero-MicroAero gas pouches and jars (Mitsubishi, Japan). Plates will be checked for growth at 3d incubation and at 7d to ensure adequate growth. At seven days incubation, colonies will be picked and mixed to form a suspension. Then the cells will be washed with phosphate buffered saline (PBS) followed by centrifugation. The supernatant will be removed and the pell will be re-suspended in 1 mL of PBS and stored in 4°C until use. Vaccine strains will be prepared no more than 1 h before use to ensure greatest stability and activity. Currently, no research lab or institution has developed a live vaccine for *C. hepaticus*. However, some companies in the US and Australia have developed autogenous vaccines but no public information is available on these vaccines. Our lab group has the requisite skills to develop live and killed strains for use in a vaccine. Once harvested, the strains will be mixed with a selected adjuvant to enhance the immune response in the host for the live vaccine. For killed vaccine, strains will be harvested as described above and subjected to treatment to kill the strains - this will consist of heat or formalin treatment, and then mixed with adjuvant for the vaccine regime, monitoring for response will be carried out by wing vein bleed and measurement of antibody levels using ELISA and IgY assays.

Vaccination and challenge chickens. Thirty-four commercially available brown hens, free of *C. hepaticus*, 16 weeks of age, will be divided into two groups (n=17 each) will be vaccinated with a live *C. hepaticus* vaccine via oral gavage. Then five sentinel birds will be added to each group to evaluate the transmission of the live vaccine. At 18 weeks of age one group that received the live vaccine will receive an inactivated vaccine and allowed to develop immunity. A non-vaccinated but challenged group (n=17) will receive a placebo orally and five sentinel birds will be added to the group. Groups will be placed in battery cages with a minimum of one square foot of floor space per hen. Housing will mimic a free-range environment similar to a large-scale layer production system. All groups, live vaccine, killed vaccine, and non-vaccinated groups will be orally challenged with *C. hepaticus* at 24 weeks of age. At intervals, post-challenge, a sub-population of vaccinated birds, sentinel birds, and challenged birds will be euthanized to collect tissues for bacterial culture and /PCR and gross lesions recorded. During the entire duration of the study, vaccine reactions, clinical signs, mortality, feed and water intake, and egg production will be recorded.

RESULTS AND DISCUSSION

The results are pending.

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A CASE OF NEUROLOGICAL DISEASE ASSOCIATED WITH AVIAN REOVIRUS INFECTION IN COMMERCIAL TURKEYS

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ABSTRACT

There have been increasing reports throughout North America of emerging presentations of various avian reovirus strains in turkeys including tenosynovitis, hepatitis, and encephalitis. A flock of 7650 four-week-old commercial turkey toms presented with 3-4% of the flock exhibiting neurological signs including torticollis, opisthotonos and ataxia. Nonsuppurative encephalitis was seen on histological examination. On PCR, brain tissue was negative for avian encephalomyelitis virus and positive for avian reovirus. The flock later developed lameness; tendon samples showed lymphoplasmacytic tenosynovitis and were positive for avian reovirus on PCR. Sequencing showed that reovirus in brain and tendon samples were most likely two different strains with 91.5% sequence identity. An increase in anti-avian reovirus antibody titers from acute and convalescent serum samples was compatible with active reovirus infection in the flock. Production impacts were severe with increased mortality and reduced slaughter weights. Vertical transmission of reovirus is suspected in this case.

INTRODUCTION

Infections with avian reovirus (ARV) are widespread in domestic birds around the world, and enteric reoviruses are common inhabitants of the gastrointestinal tract (1). Some strains of ARV are associated with runting and stunting, enteric syndromes, pericarditis, and hepatitis in birds (1). Infections with ARVs are a well-documented cause of viral arthritis and tenosynovitis in both chickens and turkeys (2–4). Reoviral arthritis and tenosynovitis is of great economic importance as it causes the birds to become lame and fail to thrive with reduced feed intake, increased feed conversion rate, and carcass condemnations (2,4). The virus can be transmitted vertically from parent flocks to their progeny and subsequently spread horizontally (1) Current prevention strategies include the use of live attenuated vaccines and autogenous vaccines which contain area-specific variant reovirus strains in broiler breeders (1,5) and autogenous vaccines in turkey breeders (6). Despite their use, disease continues to occur as new variant strains emerge and elude the vaccinal immunity (6).

CASE SUMMARY

At four weeks of age, a flock of 7,650 commercial turkey toms in Alberta, Canada began exhibiting neurological signs which included torticollis and ataxia (Fig 1A) affecting an estimated 3-4% of the flock. Nine affected birds were examined at 6 weeks of age by a poultry veterinarian. All birds were variably ataxic with some completely non-ambulatory. Most birds were sitting back on their hocks but could stand if supported. Muscle tone in the legs and wings appeared normal. Eight out of nine birds exhibited torticollis with some flexing their necks ventrally, some hyperextended dorsally, and some twisted. The birds all appeared responsive to sounds and had intact pupillary light reflexes and facial sensation. Menace response was normal to sluggish and withdrawal and knuckling reflexes were variable. On post-mortem examination, there were no gross lesions appreciated that could explain the neurological abnormalities. Three of the birds were litter-eating (>50% litter material in the gizzard), two birds had a yolk sac remnant, two birds had splenomegaly, and one bird had a mildly congested pancreas.

Pooled tissue samples and 10 sera samples were submitted to the Diagnostic Services Unit, at the Faculty of Veterinary Medicine, University of Calgary for diagnostic purposes. Histologic examination of the brain identified areas of dense cellular infiltrates in the neuropil (Fig1B) and multifocal perivascular cuffs of lymphocytes and plasma cells (Fig 1B and 1C). Spleens showed loss of white pulp and intranuclear inclusions suggestive of an adenovirus infection. Bacterial culture isolated 3 + and 2 + *E. coli* from the spleen and brain samples, respectively. Pooled oropharyngeal and cloacal swabs were negative for avian influenza virus (AIV) and Newcastle disease virus (NDV). Pooled brain sample was negative for avian encephalomyelitis virus (AEV), but positive for

ARV (Ct = 31.74). Serum samples were positive for anti-avian reovirus antibodies on ELISA (IDEXX) with a geometric mean of 4304.4±855.8 (GM±SD), n=10 (Fig 2A).

Further examination was performed when the flock was 9 weeks old, at which point the birds were no longer demonstrating neurological signs but had developed lameness. Histologically, there was moderate thickening of the tendons and synovial sheaths with a moderate number of lymphocytes and macrophages (Fig 1D&E), and rare foci of lymphocytic myocarditis (Fig 1F) were also observed in the heart. One leg had caseous exudate on gross exam and fibrinoheterophilic arthritis on histopathology. *E. coli* was isolated from that joint. Pooled tendon samples were PCR positive for ARV (Ct = 30.06).

By 11 weeks of age, the flock was exhibiting poor growth, elevated mortality, and severe lameness. On histologic examination, occasional mild lymphocytic infiltrates were recorded in the tendon sheaths. There was mild pericarditis and occasional lymphoid follicles present in the pericardium. The serum samples had anti-avian reovirus antibody titers that were increased compared to the initial samples with a geometric mean titer of 6297±1631.6 (GM±SD), n=12 (Fig 2B).

By the end of the cycle (15 weeks of age), cumulative mortality reached 15.16%, primarily due to culling of lame birds. On average, the birds only reached 70% of target body weight at slaughter. These figures represent a significant economic loss to the producer.

The ARV PCR products from the brain samples collected at 6 weeks of age and tendon samples from 9 weeks of age were further characterized by S1 gene sequencing, which encodes the sigma C protein. The viruses detected in the brain and tendons had 91.9% nucleotide sequence identity. The virus found in the brain had 94.6% nucleotide sequence identity with an avian reovirus strain from Ontario and the virus found in the tendon had 97.1% identity with an avian reovirus strain from Pennsylvania.

DISCUSSION

With the emergence of variant ARVs, new pathological presentations have been observed, including encephalitis as in this case as well as hepatitis (7,8). Recent reports from Quebec and Indiana have documented neurological disease characterized by encephalitis in turkeys with isolation of ARV strains (7,8). Similar neurologic clinical signs and histologic brain lesions have been reported in broiler chickens infected with avian reoviruses (9,10).

The flock also had histologic spleen lesions suggestive of hemorrhagic enteritis virus (HEV) infection in the first submission and a subsequent flock at that site was confirmed to be HEV positive on PCR. Because both ARV and HEV are associated with immunosuppression (1,11) it is likely that the birds were immunosuppressed due to concurrent infection of ARV and HEV. This combination is compatible with isolation of secondary invaders such as *E. coli* and higher than normal expected mortality throughout the production cycle.

The presence of lesions consistent with reovirus infection, detection of ARVs via PCR in affected tissues, and an increase in antibodies against ARV suggest that the neurological signs, lameness, and poor production in this case were associated with avian reovirus infection. The reoviruses detected in the brain and tendon samples at two different time points had a relatively low sequence identity of 91.5% and it was not possible to determine if the virus detected from the tendons at 9 weeks of age was derived as a quasi-species of the reovirus detected in brain or a new virus was introduced to the flock.

It is speculated that this case is related to other cases of avian reovirus infection in Western Canada via vertical transmission from common breeder flocks. A flock of toms with lameness, high mortality, and increased incidence of aortic ruptures was diagnosed with ARV infection causing tenosynovitis; ARV in affected tendons had a 98.2% sequence identity to the brains in the current case. Further, a case of severe reoviral hepatitis diagnosed in female poults yielded an ARV from affected livers with a 99.6% sequence identity to the virus in the tendons in the current case. Both cases were over 100 km away from the current case and may have received chicks from the same source; neither of the subsequent cases showed neurological signs.

Reporting of emerging presentations of avian reoviruses is important for recognition by producers and veterinarians and for development of control and mitigation strategies.

ACKNOWLEDGEMENTS

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(The full-length article has been submitted to the *Canadian Veterinary Journal*.)

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Figure 1. Clinical signs and histological lesions of avian reovirus infection in turkey poults. **A.** Affected bird exhibiting neurological signs with torticollis. **B**. Brain, 100x. Focus of hypercellularity in the neuropil (long arrow) and a perivascular cuff (Short arrow). **C.** Brain, 400x; perivascular cuff showing lymphocytes and plasma cells. **D.** Pale and thickened synovial membrane and tendon sheaths, 40x. **E.** Tendon sheath with edema and infiltration of lymphocytes and plasma cells, 400x. **F.** A focus of lymphocytic myocarditis in the heart, 400x.

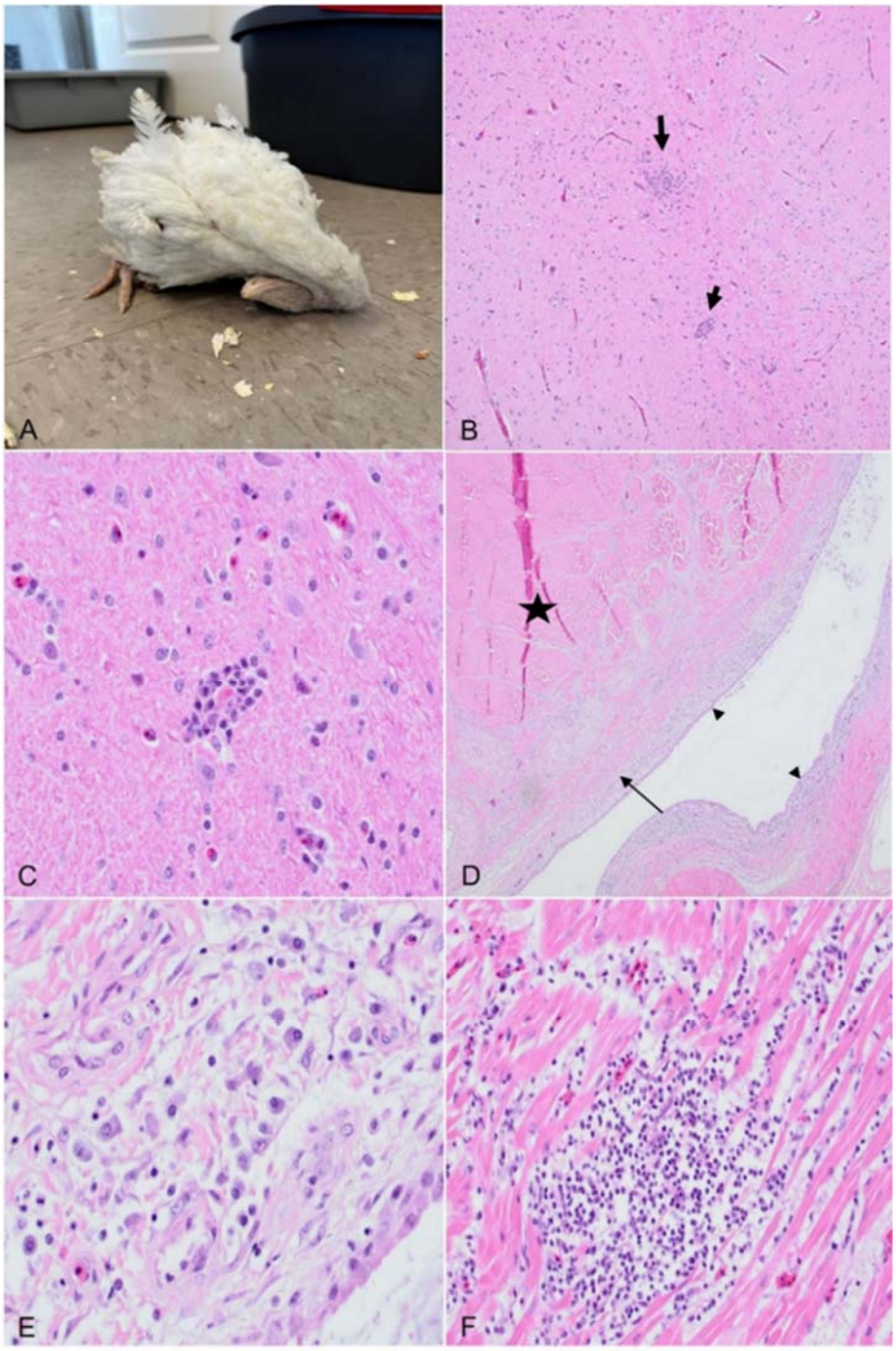
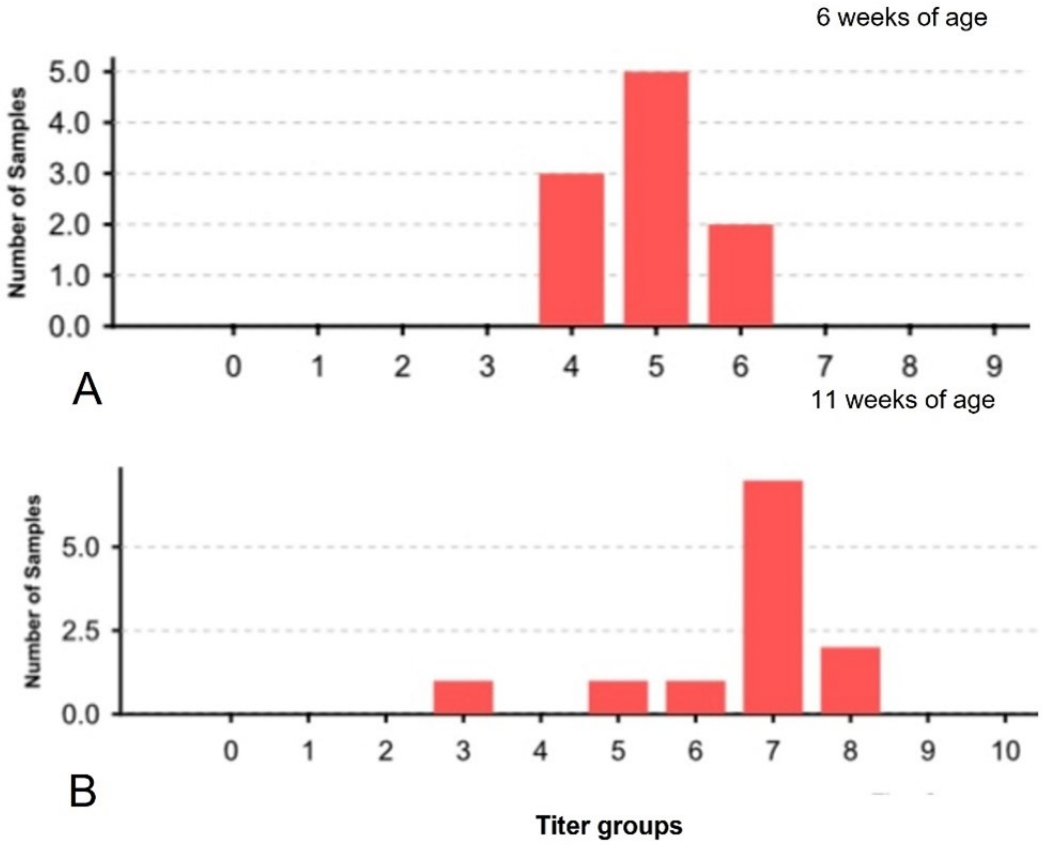


Figure 2. A. Anti-avian reovirus antibodies detected by ELISA two weeks after the beginning of the disease outbreak (six weeks of age). **B.** Antiavian reoviral bodies in turkey poults at 11 weeks of age (five weeks later).



FES2 OR AU – MOLECULAR DIAGNOSTICS VIA 3RD GENERATION SEQUENCING

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SUMMARY

Third generation sequencing or "long read" or "single molecule" sequencing is poised to become a valuable tool in molecular diagnostics for both human and animal diseases. As sequencing technology becomes more advanced, its usage in the diagnostic realm will only continue to increase. Certain best practices need to be adhered to and accepted standards agreed upon as the technology matures and is applied as a diagnostic tool. Concomitantly, when applied to diagnostics for microorganisms, baselines/backgrounds for what is considered "normal" from diverse types of samples and hosts/environments need to be determined and thresholds of believability set to help avoid chasing red herrings from results. In this presentation, the pitfalls and many promises of the technology when applied to molecular diagnostics will be explored and critically assessed to hopefully determine if third generation sequencing is fool's gold or real gold.

INTRODUCTION

Third generation sequencing or "long read" or "single molecule" sequencing is emerging as a powerful platform with possible uses in molecular diagnostics for both human and animal diseases. Due to its potential and promise, it is inevitable that a rush to apply this technology will ensue over the next few years. Cautious and thoughtful implementation will be required for DNA sequencing to yield trustworthy, accurate, and actionable diagnostic results. In this work, the pitfalls and suggested best practices for applying third generation sequencing to molecular diagnostics will be explored and critically assessed to hopefully determine if third generation sequencing is fool's gold or real gold.

Brief history of DNA sequencing. Almost 60 years ago, researchers were working on methodologies to enable the sequencing of nucleic acid molecules (1). Two different competing methods for DNA sequencing were established by Sanger and by Maxam and Gilbert in 1977 (2). Ultimately, it was the method of Sanger (dideoxy method) that prevailed and became the gold standard of DNA sequencing. In the late 1980s, automation was applied to the Sanger method, and it was this pairing that catapulted DNA sequencing into a mainstream technique in molecular biology labs around the world. It is not a stretch to believe that the remarkable success of the Human Genome project would have been delayed without such a pairing.

As the human genome project really kicked into gear, so did efforts to improve DNA sequencing. It was then, in the mid to late 90s, that Next Generation Sequencing (NGS) was born. Different DNA sequencing methodologies were invented by several companies that helped to quickly drive down the cost of sequencing a human genome from $100M in 2001 to about $10k around 2010 (3). Since the late 2000s and early 2010s, it has been Illumina® that has dominated the NGS market with their massively parallel methodology that involves sequencing by synthesis.

The next great leap in DNA sequencing began when methodologies were invented that could sequence individual nucleic acid molecules and produce much longer reads than other current NGS platforms. This single molecule sequencing that produces long reads is known as Third Generation Sequencing,

Current 3rd generation sequencing platforms. Two competing sequencing platforms represent most of the 3rd generation sequencing market, PacBio and Oxford Nanopore Technologies (ONT). Both companies introduced their different commercially viable platforms within the past 15 years, with PacBio being first to the market (4). Each platform is considered 3rd generation sequencing platforms because they both sequence single nucleic acid molecules and can produce extraordinarily long reads. In comparison to other platforms like Illumina and Ion Torrent, which produce "short" reads (limited to a couple hundred nucleotides), PacBio and ONT both can produce reads that are thousands of nucleotides long and at least for ONT, potentially millions of nucleotides long. Although, the single molecule sequencing and long reads are advantageous, one drawback to both platforms is the higher rate of miscalled nucleotides in the raw data. However, this lower accuracy is constantly improving for both platforms. With enough data and proper expertise, most effects on results can be mitigated.

Advantages/disadvantages. As with all innovative technologies, third generation sequencing has its inherent advantages and disadvantages as listed in Table 1. Most of the disadvantages are manageable if proper end user expectations are set, limitations of the data are understood, and barcoding is properly employed to help drive down the cost per sample.

Best practices. For third generation sequencing to become a routine and trustable diagnostic method, some best practices should be considered and employed in molecular diagnostic laboratories. These best practices include...

- Significant in-depth training for laboratory technicians
- Complete separation of different task areas (including reagents and equipment like pipettes) involved in the wet lab protocols
- Being aware of protocols that involve nucleic acid amplification and handling material properly
- Constantly cleaning and disinfection of all equipment and work surfaces
- Single-use aliquoting of all reagents
- Proper selection and use of positive/negative controls
- Standardization of wet lab protocols and sequence data analysis pipelines
- Understanding the potential background sequences that might arise from the usage of certain kits/methodologies and samples

Future. With 3rd generation sequencing becoming more prevalent and accessible to all labs, the future for this technology is bright. But, to reach the technology's full promise, guardrails need to be put in place if it is to become used regularly for molecular diagnostics. Some suggestions for its use and growth in the animal health arena are to put a diverse and well-versed committee in place to oversee and make decisions about what are acceptable controls, what constitutes believable results, what is just background/noise versus actual signal as it pertains to pathogens and disease, and how to ensure that labs produce accurate/consistent sequence data, plus perform analyses of that data in a correct manner that is reproducible. Along with a standing committee to help determine the aforementioned items, proficiency panels that are akin to the ones used for ELISA and PCR testing need to be produced and made available to all labs working with 3rd generation sequence data/analyses. This type of regular controlled testing will aid in adding a level of confidence to the production of sequence data and its subsequent analysis and ultimate use in a diagnostic manner. Hopefully, these steps can keep this technology from being fool's gold and propel it to becoming a gold standard for molecular diagnostics in animal health.

ACKNOWLEDGEMENTS

I would like to thank Dr. El-Attrache and Dr. Gallardo for their helpful reviews of this manuscript.

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Table 1. Comparison of the advantages and disadvantages for 3rd generation sequencing.

Advantages	**Disadvantages**
Test for multiple pathogens at one time	Higher error rate than other NGS methods
Detection and characterization can be performed simultaneously	Costly if samples are not batched and barcoded
Not required to know exactly what pathogen one is looking for in any given sample	Limit of detection is not equivalent to qPCR or RT-qPCR in most cases
Many public software tools exist for creating highly automated pipelines for data analysis	Wet lab protocols require highly skilled laboratory technicians
Potentially portable for field testing (ONT)	Data analysis requires skilled bioinformatician
Methodologies can be designed that are less prone to fail if microorganisms mutate	

GENETIC ANALYSIS OF LOW PATHOGENIC NEWCASTLE DISEASE VIRUS (LONDVS) ISOLATED FROM WILD BIRDS DURING ADAPTATION TO CHICKEN EMBRYOS

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INTRODUCTION

Newcastle disease, caused by the Newcastle disease virus (NDV), is one of the most economically important diseases for poultry production worldwide (Miller *et al*., 2010). The periodic occurrence of NDV vaccine strains in wild birds in North America indicates that transmission from domestic birds to wild birds is possible (Ayala *et al*., 2016). NDV may also be transmitted back from wild birds to chickens (Brown *et al*., 2017; Bello *et al*., 2018; Habib *et al*., 2018; Ferreira *et al*., 2019; Abd Elfatah *et al*., 2021). This study investigated the adaptation of NDV strains from aquatic birds to the chicken as host and if that might cause an increase in virulence for chickens.

MATERIALS AND METHODS

Six isolates of low-pathogenic Newcastle disease virus (loNDV) isolated from wild aquatic birds were passaged in chicken embryos for ten passages. The virulence of the first and the last passage of each isolate was compared by embryo mean death time (EMDT). The whole genomes of the first and tenth passages were sequenced using Illumina. For each isolate, the genome of passage 1 was assembled de novo and the reads of passage 10 were aligned with the passage 1 genome for variant calling. Variant functional consequences were predicted using the Ensembl VEP software. Phylogenetic analysis was done to determine evolutionary relationships among isolates. The phylogenetic trees, based on complete genome sequences, were performed using the neighbor-joining method with 1000 bootstraps in MEGA software (Tsunekuni *et al*., 2010; Wang *et al*., 2016; Dimitrov *et al*., 2019).

RESULTS

There were only minor differences between EMDT of the first and tenth passage with no recognizable trend. Sequences covering the full genome sequences >15 kbp in length were obtained from both passages of all six isolates. The mean sequencing depths were between 95 and 480. Preliminary analysis showed that close to 300 single nucleotide polymorphisms (SNPs) and almost 20 INDELs were present in all isolates. The number of SNPs was between 34 and 71 in each isolate and the number of INDELs between 1 and 6 in each isolate. Phylogenetic analysis of the whole genomes identified isolates 5, and 8 among the Class I NDV and isolates 6, 7, 9, and 10 among the Class II NDV.

CONCLUSION

This study demonstrates how loNDVs from aquatic birds adapt to chickens as host. These identified variants are likely to play a role in the adaptation to chicken embryos, however, there is no indication that the virulence for chickens increased.

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STUDY INVESTIGATING THE IMPACT OF LATE IBDV CHALLENGE ON DMV/1639 PROTECTION IN COMMERCIAL BROILERS BASED ON IBV AND IBD VACCINATION STATUS

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INTRODUCTION

Infectious bursal disease virus (IBDV) infections within the first two weeks of age cause significant immune suppression while uncomplicated infections after three weeks are less severe and more transient. Both IBDV and IBV field infections commonly occur between three to four weeks of age. The purpose of this study was to see if a three-week IBDV challenge could significantly reduce immunity against an infectious bronchitis virus (IBV) challenge given 4 days later. A secondary objective was to see if vaccination with a recombinant IBD vaccine (rHVT-IBD) could minimize any negative impact AL2 challenge might have on IBV protection. IBV infection levels and lesions were assessed in both IB vaccinated and unvaccinated birds. AL2 is the most prevalent IBD field virus in the United States. Massachusetts and GA08 vaccines are the most common commercial IB vaccine serotypes used together to help control DMV/1639—the most prevalent IBV field challenge type we face today.

MATERIALS AND METHODS

Three hundred twenty-four straight-run commercial Ross 708 chicks were randomly assigned to six treatments (see table), neck tagged, and placed at 18 per isolator according to a completely random design (three isolators per treatment). 15 non-IBD vaccinated chicks were bled for IBDV serology to determine maternal immune status. Birds in treatments 5/6 were *in ovo* vaccinated with rHVT-IBD and treatments 1/3/5 were vaccinated with Mass + GA08 vaccine by coarse spray cabinet (14 mL/100 chicks) on day of hatch. At 21 days of age, birds in treatments 3/4/5/6 were inoculated with 3.5 EID50 AL2 IBD virus (.04 cc each in eye and nostril). On D25 all treatments were inoculated with 3.5 EID50 DMV/1639 IB virus. On D31 trachea tissues were collected from all the birds for IBV PCR (upper quarter) and for histopathology (lower 2/3 of trachea). All hypotheses were conducted at the $p \leq 0.05$ level of significance with the Shaffer simulated method used to adjust for multiple comparisons.

RESULTS

Day of age IBD ELISA serology (Idexx IBD-XR) yielded a respectable 7,480 GMT and 45% CV. However, the 21-day AL2 challenge did result in significant bursal atrophy (T1/2 = **5.83** vs. T3/4/5/6 = **4.33**) using bursameter scores. The DMV/1639 challenge gave a high take rate with 97-100% IBV positive (<Ct-35) in non-vaccinated treatments T2/4/6. IB vaccination resulted in significant IBV protection based on all measured parameters—mean viral loads and lesions and percentage of birds protected against infection and lesions. However, IB vaccinates that were AL2 challenged prior to DMV/1639 challenge (T3) were significantly less protected than controls (T1) based on mean Ct-value, mean tracheal thickness, protection from infection and histological lesions. In contrast, IB vaccinates that also received a recombinant HVT-IBD vaccine (T5) were spared this reduction in acquired IB protection caused by the AL2 challenge.

DISCUSSION

Few people would dispute that flocks infected with IBDV by two weeks of age carry a high risk of becoming significantly, if not permanently, immune suppressed. However, opinions about the significance of IBDV infections after two to three weeks are much more mixed. At best there is minimal to no impact if there are no other stressors to the immune system and at worst the immune suppression can be significant but more temporary in nature. In this study, a three-week AL2 challenge reduced the immunity acquired from day of age IB vaccination, measured by protection after a 3-½ week IBV challenge. These lower protection levels given by day of age IB vaccination when there was an AL2 challenge were sometimes significant and other times only numerical—and showed that the acquired IB immunity was at least temporarily reduced. Finally, treatment 5/6 results

show the potential of rHVT-IBD vaccination to minimize any transient dip in immunity caused by a late IBD infection. In short, this study supports the growing trend of using recombinant HVT-IBD vaccines even when breeder IBD programs are solid—especially during winter respiratory season when both IBV and IBDV levels are typically at their highest.

Table. DMV/1639 IB immune status based on hatchery vaccination status and late AL2 challenge

Treatment	Vaccination Status		Challenge		Mean Ct-value	Tracheal **% Protection** from:			Mean mucosal thickness
	IBD *in ovo*	IB coarse spray at hatch	IBD at 21 days	IB at 25 days		Ct-35 level infection	Histo-logical lesions	Mucosal induration (thickness)	
T1	-	Mass+GA08	-	DMV	37.4A	74A	90A	73	59.5
T2	-	-	-	DMV	27.6	0	5	19	93.4A
T3	-	Mass+GA08	AL2	DMV	34.7B	30B	55B	61	69.1
T4	-	-	AL2	DMV	27.2	0	5	12	114.7B
T5	rHVT-IBD	Mass+GA08	AL2	DMV	35.8AB	57AB	90A	70	61.7
T6	rHVT-IBD	-	AL2	DMV	28.4	3	5	29	92.2A

- IB vaccinated treatments (T1/3/5) were significantly different than non-vaccinated treatments (T2/4/6) on all IBV parameters.
- Within IB vaccination treatments (T1/3/5), AL2 challenge significantly reduced protection against Ct-35 level infection and histological lesions unless they also received rHVT-IBD (T5).
- Within non-IB vaccinated treatments (T2/4/6), AL2 challenge significantly increased mucosal thickness unless birds also received rHVT-IBD (T6).

COMPATIBILITY STUDY OF LIVE ST AND *E. COLI* VACCINES WHEN CO-ADMINISTERED WITH IB VACCINES BY SPRAY POST-HATCH BY MEASURING IBV TAKES (PCR)

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INTRODUCTION

Massachusetts and GA08 are the most commonly used infectious bronchitis (IB) serotype vaccines given by day of age spray in the broiler industry—especially in combination to help cross protect against DMV/1639 field challenge. IB Mass vaccine has also become commonly applied by day of age spray in commercial pullets to help minimize risk of false layer syndrome caused by early IBV field challenge. Live *Salmonella* Typhimurium (ST) and *E. coli* vaccination is very common in long lived birds and even practiced in broilers when challenge levels and risk justify the additional vaccine cost (1,2).

A previous study demonstrated that co-administering live ST and *E. coli* vaccines by coarse spray at either day of age or 2 weeks did not compromise protection against a wild type ST (multi-drug resistant DT-104) nor against an avian pathogenic *E coli* (APEC) challenge given at 6 weeks of age (3). Another study previously demonstrated that live *E. coli* vaccine was compatible when co-applied by day of age coarse spray with a combination ND/IB vaccine, based on protection against APEC as well as velogenic Newcastle disease virus (NDV) challenge (4). This is the first time we've studied the compatibility of the two live bacterial products on IBV takes when either or both were co-administered with Mass and GA08 vaccines by day of age coarse spray.

MATERIALS AND METHODS

Two hundred forty Ross broiler chicks were randomly assigned to four different vaccine treatments: 1) IB vaccine only (Mass + GA08), 2) IB plus live *E. coli* vaccine, 3) IB plus live ST vaccine, and 4) IB plus live *E. coli* and ST vaccines. Birds were then placed 30 per isolator according to a completely random design (two isolators per treatment). Birds were vaccinated by coarse spray cabinet (14cc/100 birds). On four, five, and seven days of age, tracheas were collected from 10 birds selected by first grab from each isolator (20 per treatment) to be processed for IBV PCR at Zoetis. All hypotheses were conducted at the $p \leq 0.05$ level of significance with the Shaffer simulated method used to adjust for multiple comparisons.

RESULTS

The IB vaccine percentage takes (<Ct-35) were high for both vaccine serotypes (95% for Mass and 97% for GA08, overall) by four days of age, with or without the addition of one or both live bacterial vaccines. In fact, only one of the 80 birds in the four-day sampling was completely negative on Mass IB PCR. The mean Ct values for Mass were at their lowest (strongest) at five days. The GA08 % positive takes were high in all groups at all three sampling windows but the mean Ct values tended to be a little lower at five-seven days.

DISCUSSION

Previous studies have demonstrated that the co-administration of live *E. coli* and ST vaccines by coarse spray at either day of hatch or two weeks of age does not diminish the immune response to either. The co-administration of the live *E. coli* vaccine with a combination live ND/IB vaccine by day of age coarse spray also did not compromise immunity to avian pathogenic *E. coli* or velogenic NDV. While the current study did not test actual IBV protection, the fact that the take response to the two IB vaccine serotypes was not affected in any measurable way when co-administered with either/both live bacterial product lends support to what has been commercially practiced already for years.

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Table. IB PCR results at different days after spray vaccination with Mass + GA08 +/- live bacterial vaccine(s).

Treatment	**Live bacterial product added to Mass + GA08**	**Mean Ct values followed by percentage of birds with a Ct-value below 35.0**					
		4 Days of Age		5 Days of Age		7 Days of Age	
		Mass	GA08	Mass	GA08	Mass*	GA08
T01	None	27.7 (93)	29.2 (88)	24.5 (100)	25.5 (98)	31.7 (80)	24.1 (100)
T02	Live *E. coli*	26.1 (100)	27.7 (100)	23.0 (100)	28.3 (100)	31.0 (93)	25.0 (100)
T03	Live ST	30.0 (90)	25.8 (100)	25.6 (100)	24.1 (100)	32.0 (80)	24.2 (100)
T04	Live *E. coli* and Live ST	29.6 (95)	25.9 (100)	26.8 (98)	25.7 (100)	30.7 (80)	25.0 (95)
Total Positive (<Ct-35)		94.5%	97.0%	99.5%	99.5%	83.3%	98.8%

*At least 95% of birds in each treatment had a positive IB Mass signal (<Ct-40) at 7 days.
There were no significant differences between any treatments at any time points.

THE CYTOLOGY OF AVIAN MONOCYTOSIS – A LAYING HEN PERSPECTIVE

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INTRODUCTION

Monocytosis is a disease of pullets and turkeys affecting egg production and general wellness. Long known by a variety of common names, blue-comb, and pullet disease are but a few of its many sobriquets. Various causes have been implicated, including nutrition and infectious agents such as coronavirus (1). Description of the blood picture, particularly the condition of cells in the monocyte series can aid in the diagnosis. Leukocytosis detected at 56 wk in LSL hens was (TWBC ~85K/µL) for samples from conventional (CC), enriched (EN), and aviary (AV) cages. SDC monocytes (Mn) at AV (13%) CC (7%) EN (8%) indicated monocytosis. Bacteria and various fungal forms were seen in nearly all blood samples. Some of the cytological atypia were likely due to microbial toxins. An example of monocytosis in a wild Mallard is included for comparison.

MATERIALS AND METHODS

Blood, stain procedure and microscopy. Whole blood was drawn from the brachial veins of LSL hens at 56 wk into EDTA tubes. The Mallard sample was a touch preparation made at capture/release. Staining was by Wright's method followed by a brief secondary exposure to Giemsa. Photos were obtained with an Olympus CX-41 light microscope at either 40x or 100x (oil); image capture was with an Infinity-2 1.4-megapixel CCD USB 2.0 Camera. Photo processing was with Infinity Analyze (Release 6.5) software.

RESULTS

Examples of reactive mixed monocyte emboli in 56 wk LSL hen blood are in Fig. 1 A; left-shift heterophils are integral to the aggregate - some Mn's display secretory (plasmacyte) features. A naked Mn nucleus is located at N. A reactive Mn giant cell is in Fig. 1B. A mitotic Mn is located at M (aspindylosis). A reactive (net type) basophil (Ba) (2) and a toxic ghost heterophil are with transitional mono/plasmacytoid types in Fig 1C. A giant blast (Türk, proplasmacyte) cell has a conspicuous Hof and large nucleolus [Cell area 141 µm^2, A_N (area nucleus) 79 µm^2, Ploidy Ratio 2.6] (3) is of tetraploid/hexaploid size. This cell is typical of others in the study (Fig. 1D). Monocytosis of a wild Mallard (2 yr, Female) is in Figure 2A. A pair of reactive Mn is in the company of reactive Ls. Fig 2B. 3 Reactive Mn are anchored by a central Lm. Fig 2C. A portion of a giant Mn embolus showing transitional Mn. Fig 2D. A macrogamete of *Haemoproteus sp*. from the same sample. Fig 2E. A mycelial form of *Hemomycetes avium* (3) is also from the same sample.

DISCUSSION

Atypical cells of the monocyte series were found at levels high enough to support a retrospective diagnosis of monocytosis occurring at 56 wk in LSL hens housed 3 cage styles. Similar cytology was found in a wild Mallard. Moreover, the present results demonstrate atypical behaviors of reactive Mn cells. Included are giant polyploid cells, mitotic cells, a tendency to shed cytoplasm, and cells with transitional lymphoid/monocytoid phenotypes like those earlier described for turkeys (4). These observations have not been reported elsewhere and may be helpful when considering a diagnosis of monocytosis. As cytological observations of monocytosis are scarce these supply a need.

ACKNOWLEDGEMENT

The blood samples were from the Coalition for a Sustainable Egg Supply (CSES) project.

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Figure 1. A. Left-shift heterophils & Mn's displaying secretory (plasmacyte) features. N, a naked Mn nucleus. B. A reactive Mn giant cell. A mitotic Mn is located at M (aspindylosis). C. A reactive (net type) basophil (Ba) and a toxic ghost heterophil are near transitional mono/plasmacytoid types. D. A giant blast (Türk, proplasmacyte) cell has a conspicuous Hof and large nucleolus.

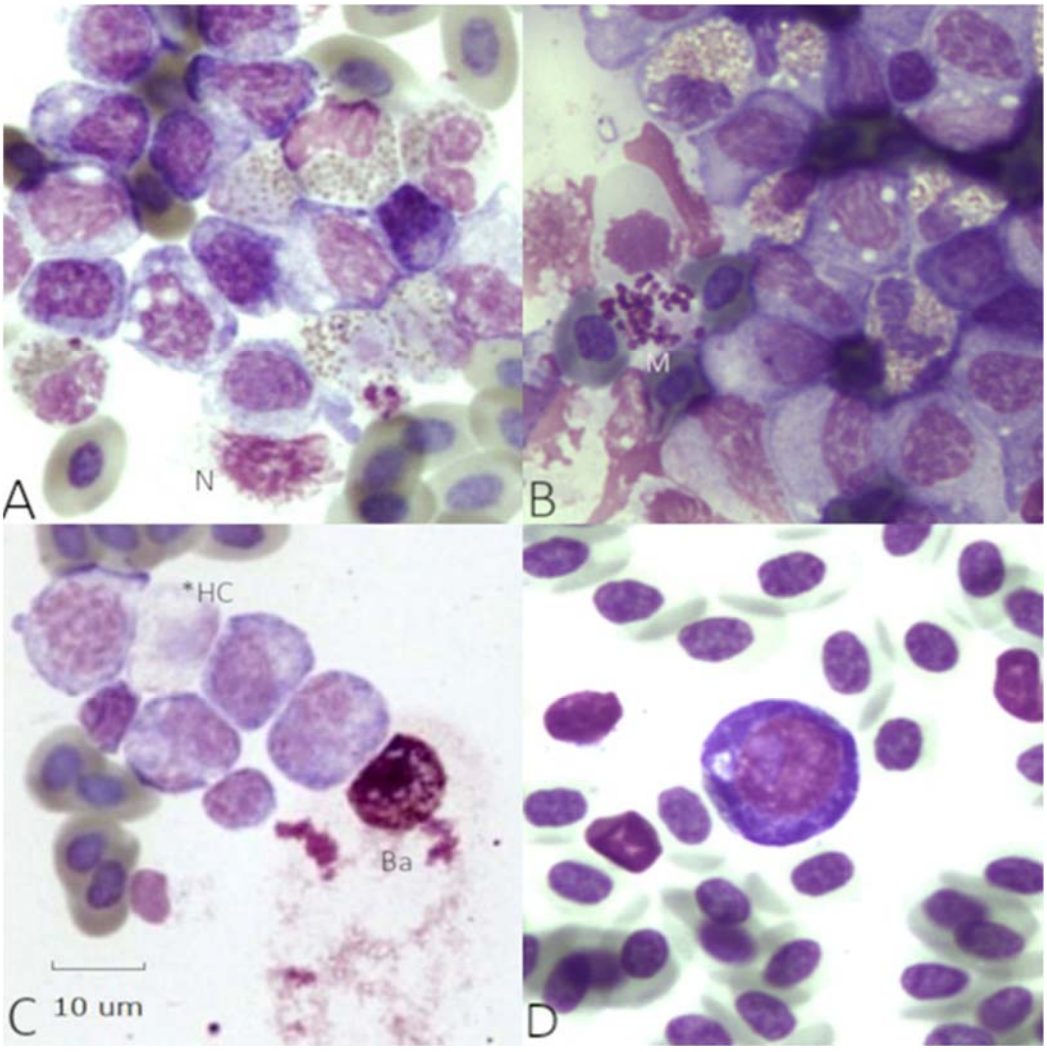
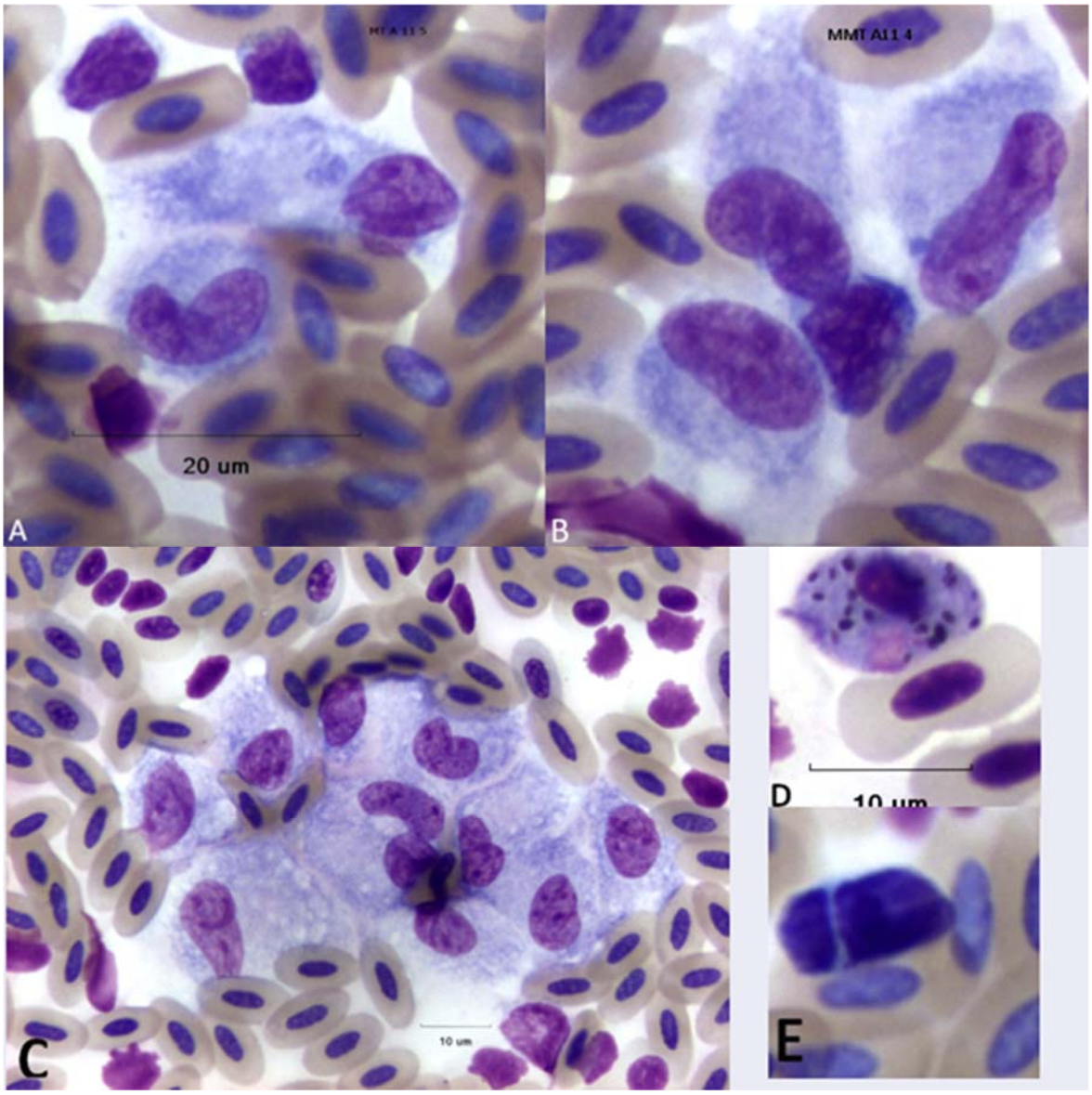


Figure 2. Protozoan and fungal infections accompany monocytosis in a wild Mallard. A-C Reactive Mn and lymphocyte emboli, Mn show transitional characteristics. D. *Haemoproteus* macrogamete, and E. *Hemomycetes avium* mycelial form in the same duck.



AVIAN POLYCLONAL B-CELL LYMPHOCYTOSIS – A STRESS INDICATION OR A CONSEQUENCE OF INFECTION?

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INTRODUCTION

Polyclonal B-cell lymphocytosis is a benign dyscrasia originally described in human female smokers. Circulating B-cells with atypical morphological features (bi-nucleate) and IgM hypergammaglobulinemia are its characteristics (1). In some cases, a chromosomal abnormality i(3q) is also seen, as is the signature cytological feature, binucleated plasmacytes (binPC). As binPCs suggest viral infection the purpose is to describe this type of atypia as found in experimental (Marek's disease) Leghorn chicks.

Blood, stain procedure and microscopy. Whole blood was drawn from the brachial veins of experimental Leghorn chicks, spread onto slides, and fixed in 100% MeOH. Staining was by Wright's method followed by a brief secondary exposure to Giemsa. Photos were obtained with an Olympus CX-41 light microscope at either 40x or 100x (oil); image capture was with an Infinity-2 1.4-megapixel CCD USB 2.0 camera. Photo processing was with Infinity Analyze (Release 6.5) software.

RESULTS

Examples of binPC in the blood of Marek's challenged, and vaccinated experimental Leghorns at various ages are in Figure 1. Those whose nuclei are ~ equal in size are mitotic products unless otherwise indicated. The binPC cytoplasm is patchy due to ER distention and some cells have clear Hofs; characteristics of plasmacytes. A skeletal SDC result that accompanies the figures is in Table 1. Additional examples of binuclear atypia including a bin basophil from 1 blood film are given in Figure 2.

CONCLUSIONS

Collectively the observations demonstrate the occurrence of binuclear leukocytes in the blood of experimental Leghorn chicks. The binuclear condition appears to arise from both mitotic and amitotic cell division as indicated by both equal and unequal daughter nuclei size. This condition can occur in the absence of leukocytosis or stress as determined by the H/L ratio. It occurs at diploid and higher ploidy levels (2). The diverse cytology described here supports polyclonality. It may be a useful indication of (viral) superinfection.

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Figure 1. A. An isolated binPC appears in a field of mature RBCs. The cell size AC ~ 88 µm2 is ~ equal to heterophils. The nuclei [AN 32 µm2 and 25 µm2] are likely diploid, with patchy (cobblestone) chromatin; no nucleoli are visible. B. A binPC is to the left in a field with a thrombocyte (Th) a variant heterophil (HV) and several small encapsulated bacteria (near arrow). The nearly equal reinform nuclei [AN1 25 µm2, AN2 20 µm2] display the characteristic cobblestone heterochromatin arrangement, and are diploids. C. A prebinPC at early amitosis. D. Two binPC appear in a field with small [AC ~30 µm2] lymphocytes (Ls, T-cells). The separating nuclei are at the early isthmus stage of amitosis and of unequal size [AN1 34 µm2; AN2 16 µm2 (hypodiploid)].

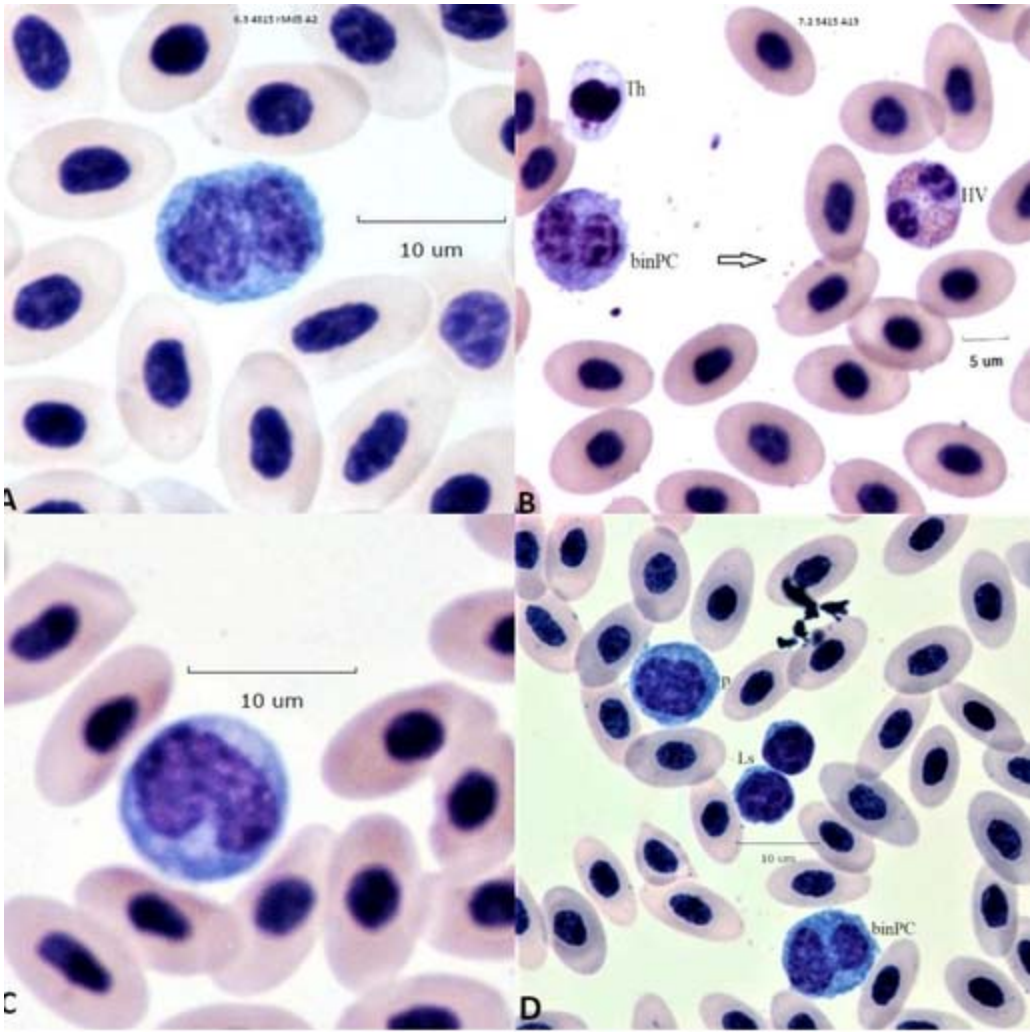
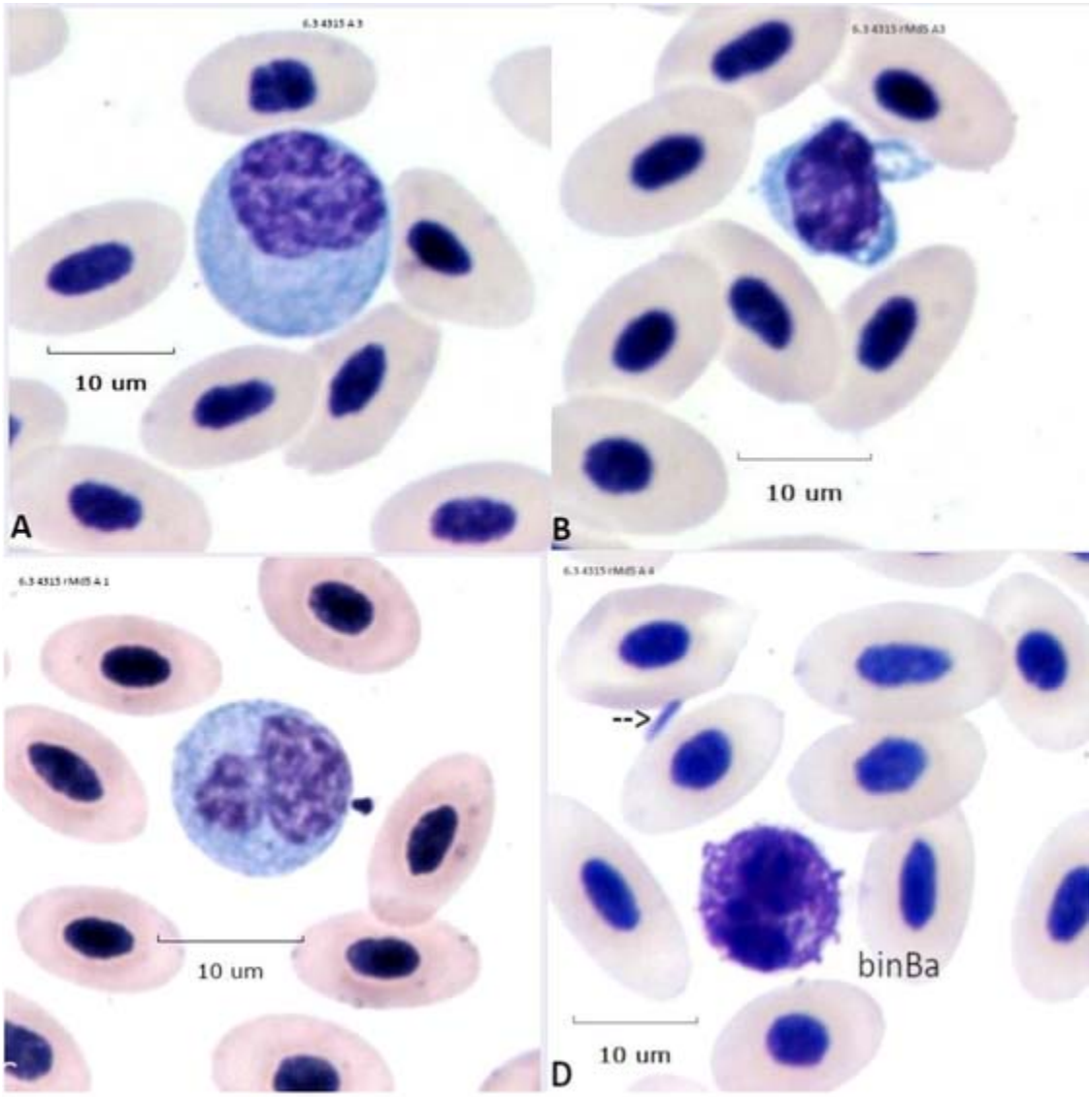


Table 1. Skeletal SDC for Figure 1.

Panel	Trt	TWBC(K)	H/L
A	MD1.	20	0.16
B	Ctrl	25	0.23
C	MD	40	0.33
D	Vac	50	0.33

Figure 2. A. A large [AC 227 µm2] mononuclear polyploid plasma cell [Ploidy Ratio 3.3; diploid ~1.6]. B. A reactive Lm with pseudopods. C. Asymmetric binuclear plasma cell [AC 102 µm2]. D. Binuclear (mitotic) basophil in a field with an RBC cell-associated bacillus (arrow).



USE OF AN AI-POWERED MICROBIOTA ANALYSIS PLATFORM (GALLEON™) AS A TOOL TO EVALUATE FIELD INTERVENTIONS IN BROILER CHICKENS

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SUMMARY

Microbiota analysis data from broiler chickens under research and field-testing conditions have become more prevalent over recent years. However, the relationship between microbiota composition and effects on broiler performance or disease mitigation has been challenging to establish. This article will focus on a practical, microbiota-based, non-invasive AI (artificial intelligence)-powered tool (Galleon™ Microbiome Assessment Platform) that allows the analyses of complex big data sets and turns them into actionable insights. This paper also describes how Galleon can be used to support commercial production of broilers by deriving insights from the interaction between the chickens' gut microbiota and pathogens such as *Campylobacter*.

INTRODUCTION

The intestinal microbiota plays a crucial role in chicken health and production performance (1, 2). The maturation of the microbiota of chickens includes rapid successional changes, developing from a simple to a more complex and diverse composition based on gradual colonization with microbiota (3, 4). Delaying or disrupting this development pattern as caused by high antibiotic use or poor chick quality, for example, results in lower performance and increased pathogen risk (5). Different interventions such as probiotics, prebiotics, postbiotics, phytogenic compounds, and organic acids in addition to formulating diets low in fermentable protein have been shown to promote early microbiota maturation in broiler chickens (6).

For that reason, microbiota analysis data from broiler chickens under research and field-testing conditions have become more prevalent over recent years. However, analysis of the gut microbiota by molecular approaches has identified bacterial populations of over 600 species from more than 100 genera. The relation between bacteria, and between bacteria and factors impacting them may also be non-linear which makes the relationship between microbiota composition and effects on broiler performance or disease mitigation challenging to establish (7, 8).

After a decade of development work, using data from numerous broiler trials testing AI models, Cargill created Galleon, a practical non-invasive microbiota analysis tool that can be used to determine how the flock gut microbiome is related to their nutrition and health, and management practices. In the Galleon procedures, the microbiota of cloaca swab samples undergo assessment via a microarray chip with previously selected DNA populations (biomarkers), which are then analyzed using statistics and non-linear AI models.

Galleon has been used since 2016 and has accumulated data from more than 44,400 samples collected and analyzed. The main purpose of using the platform has been comparing microbial profiles amongst different production conditions such as nutritional or health programs, additives, management practices, and to establish microbiome health monitoring programs. All these information and insights will be used to support decision making, understand specific pathogen risk linked to microbial profiles, reinforce field trials on risk assessment besides clinical evaluation and assist internal R&D activities in research farms or field trials.

This paper describes a field case where a broiler producer was facing a recurrent issue with increased *Campylobacter jejuni* incidence, as identified by veterinary surveillance. Galleon allowed the analysis of complex big data sets providing valuable insights into the interaction between the chickens' gut microbiota, pathogens, and different interventions targeted to reduce pathogen incidence.

MATERIAL AND METHODS

Galleon was used to investigate the relationship between broiler farms with high and low incidence of *C. jejuni* and their gut microbiota. The results of this assessment were used to design 2 interventions to reduce *Campylobacter* incidence in high-risk farms. Two broiler (ROSS 308, mixed sex) farms in each category (four in total) were selected based on historical scores defined by the local veterinary service and classified as Positive or Negative based on *C. jejuni* risk and followed for three different cycles. In the first cycle the microbiota profiles of the two positive and two negative farms were compared using cloaca swabs collected from 24 broilers from each flock at 7 and 21 days of age and analyzed based on Galleon protocol. In the second cycle, the effect of a Probiotic (applied in the hatchery) on the microbiota profile of positive and negative flocks of the corresponding farms was evaluated. Cloaca swab samples were again collected from 24 broilers from each flock at 7 and 21 days of age. In the third cycle, the effect of combining the same probiotic (in the hatchery) with a Prestarter diet on the microbiota profile of the same farms was evaluated. Cloaca swab samples were once again collected from 24 broilers from each flock at 21 days of age.

Fluorescence readings of the microarrays that passed data quality control were used to calculate relative intensity data for each bacteria DNA probe. After normalization, data were subjected to ANOVA in a factorial arrangement with fixed effect of sampling round (Baseline, Probiotic or Probiotic+Prestarter), farm pathogen class (Negative vs. Positive), age (7 and 21d) and their interaction. Pairwise comparisons between standardized LS-means were made for each bacterium and differences considered significant by passing a FDR (False Discovery Rate) test with P = 0.05. Results were used for cluster analysis of significant bacteria and to create volcano plots to show outcomes for each pairwise comparison. Effects were also used to create AI models, including the selection of the most important bacteria for the significant models.

RESULTS

In the heat map (Figure 1) from the first cycle (positive x negative farms), farm classes and age were grouped based on similarity on vertical clusters while bacteria are grouped based on similarity horizontally. Treatments were clustered first by class, then by age. At 7 days, samples of the Negative class had a higher signal of cluster 5 rich in *Lactobacillus,* and lower signal of bacteria in clusters 1, 2, 3 and 4 which included *Streptococcus* (cluster 3), lower gut fermenters (*Bifidobacterium*, *Lachnospiraceae* and *Faecalibacterium*) but also proteolytic bacteria (cluster 1). At 21 days, samples of the Positive class showed a higher signal of cluster 3, which included *Campylobacter jejuni* and *Streptococcus,* while the Negative class was associated with cluster 4 which included desirable bacteria such as *Lachnospiraceae, Ruminococcus* and *Faecalibacterium.*

At 7 days the pathogens *Enterococcus* and *Salmonella* were significantly higher for the Positive class in association with higher *Streptococcus,* although there were no significant differences for *Campylobacter* at this age. The Negative class was linked to higher *Enterococcus hirae*, *Serratia marcescens*, and *Escherichia coli*. At 21 days, *Campylobacter* was higher in the Positive class in contrast with higher *Alistipes*, *Lachnospiraceae*, *Ruminococcus*, and *Faecalibacterium* in the Negative class (Figure 1).

During second cycle (intervention with a probiotic on all flocks) at 7 days, the Probiotic induced a clear shift towards *Lactobacillus* in the Positive class compared to the Baseline, which is an indication of a better start of the microbiota.

The volcano plots of Figure 2 compare microbiota of the Positive flocks before interventions (first cycle) with the second cycle (effect of a Probiotic on positive and negative flocks) and third cycle (effect of combining the Probiotic with a Prestarter). At 21 days, the Probiotic induced a shift from high *C. jejuni*, *Streptococcus*, *Lachnospiraceae*, and *Bifidobacterium* in the Positive Baseline towards *E. coli*, *E, hirae*, *Citrobacter*, *Faecalibacterium*, and *Alistipes* (Figure 2, left). Probiotic in combination with the Prestarter significantly reduced *C. jejuni*, but also reduced *E. coli*, *Citrobacter*, and *E. hirae* signals compared to the Probiotic alone (Figure 2, right). The benefit of feeding the Prestarter was confirmed by the AI model comparing the first and third cycle for the Positive flocks. This model was highly accurate (96%) and statistically different from random (p=0.01). The AI model comparing Baseline and Probiotic had a 86% accuracy but was not statistically significant (p=0.13).

DISCUSSION

At seven days, *Lactobacillus* is a dominating species in normal microbial development as measured by Galleon™ Microbiome Assessment Platform. Their reduced presence in Positive farms indicated an impaired early microbial development, less able to keep proteolytic bacteria under control by competitive exclusion. This can be a predisposing gut environment to the rise of bacteria such as *Enterococcus*, pathogenic *Clostridium*, and *Salmonella*. Promoting *Lactobacillus* in the first week instead of

Streptococcus in Positive farms, demonstrated to be beneficial by reducing proteolytic bacteria, including *C. jejuni.*

Application of the Probiotic in the hatchery significantly improved the composition of the microbiota resulting in a reduction of *C. jejuni*. This indicates the importance of the presence of good bacteria as first colonizers to prevent pathogen establishment in the gut. However, the effect of combining the Probiotic (in the hatchery) with a Prestarter diet on farm created an even better microbiota profile with a reduction of other proteolytic bacteria such as *Citrobacter* and *E. coli*.

CONCLUSION

Galleon is a practical, non-invasive microbiota analysis tool that allows the study of interactions between host, environmental factors, and the gut microbiota. The insights generated by Galleon are useful to assess and monitor pathogen risk, to unravel the pathogen - gut microbiome relationships, to design and evaluate interventions to reduce pathogen risk, and further to develop novel solutions.

In the presented case it was possible, through Galleon insights, to promote microbiota maturation and steer the microbiota towards a more stable and healthy state that has shown to result in reduction of *C. jejuni*, *Citrobacter*, and *E. coli*.

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Figure 1. Cycle 1, Microbiota differences between Positive (P) and Negative (N) classes at 7 and 21 days.

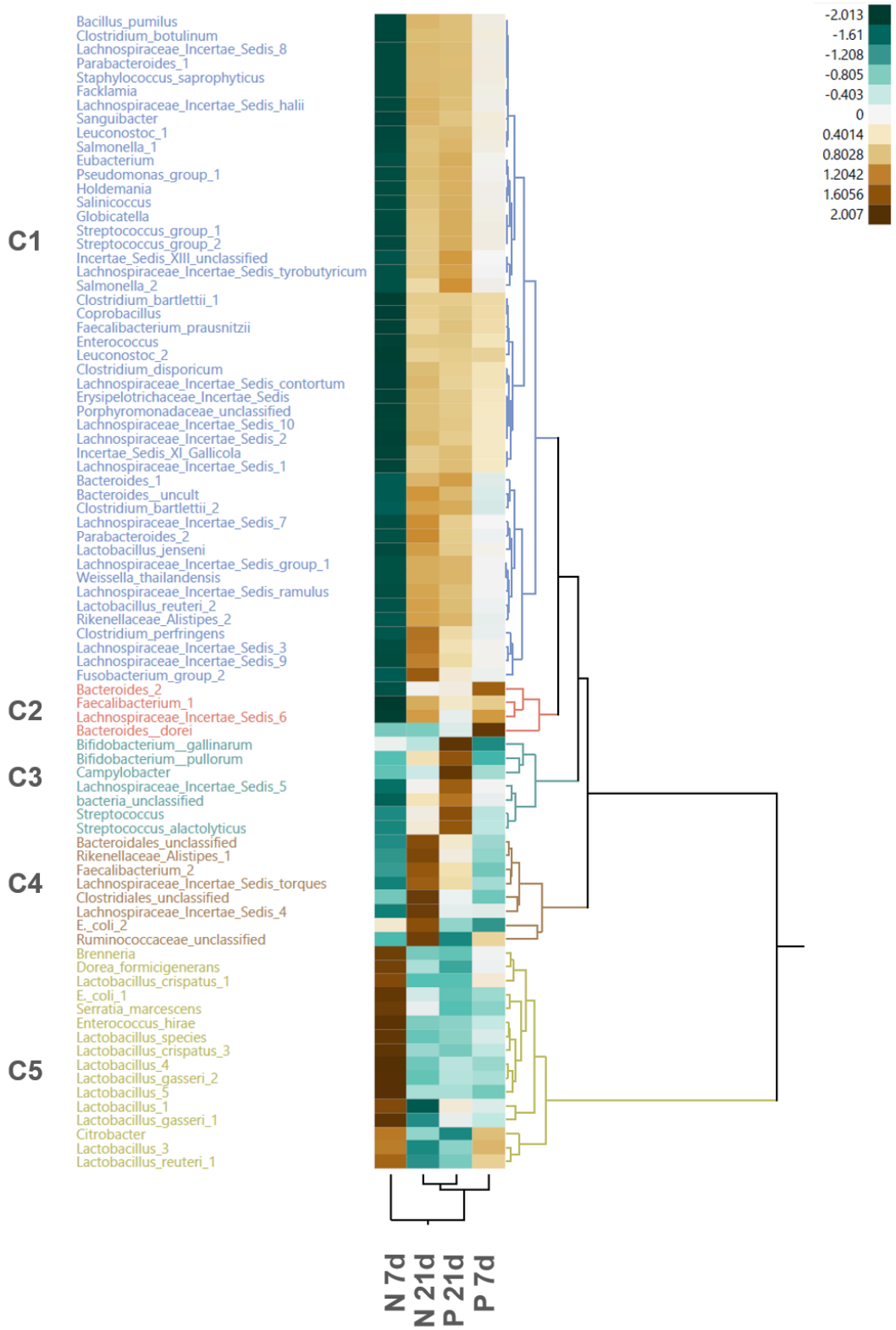
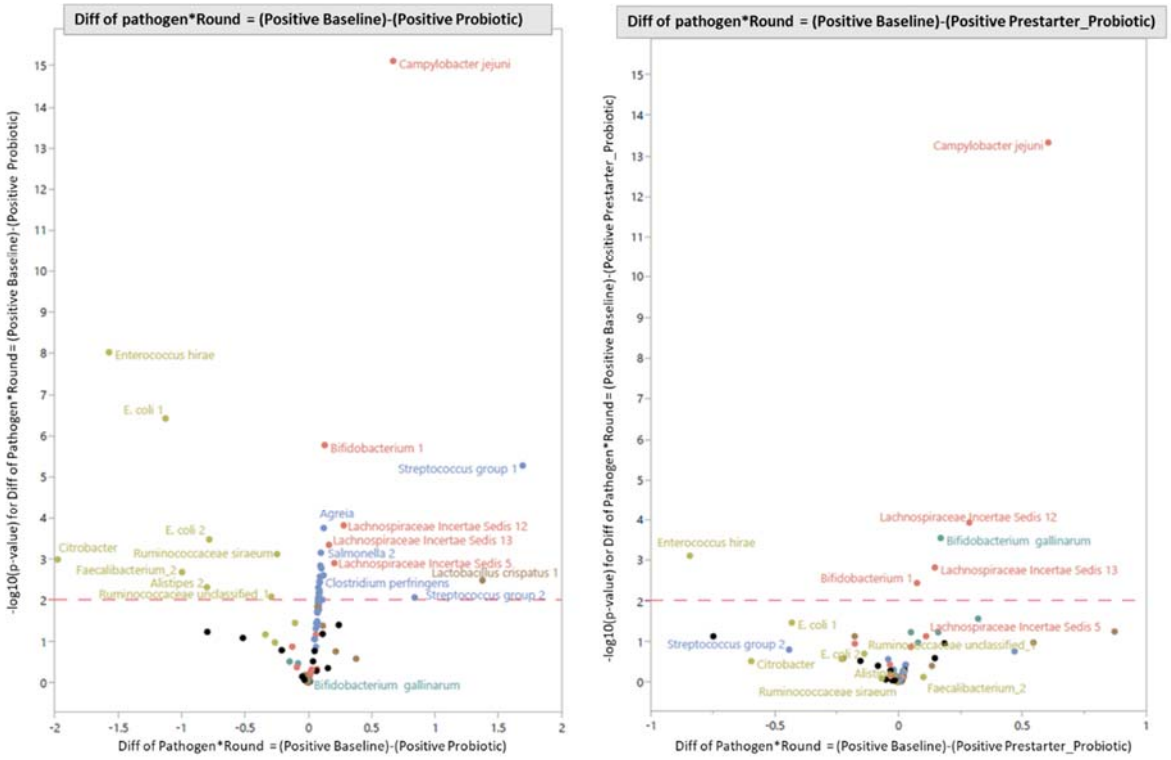


Figure 2. Comparison between Cycle 2 and Cycle 3 Volcano plots[1] for the pairwise comparison of microbiota differences between Positive Classes with and without Probiotic (left) and with and without Probiotic + Prestarter (right) at 21 days.



[1]This plot represents a subtraction, if the abundance is higher for the first factor (positive baseline), the number will be positive and placed on the right side of the zero. If higher for the second factor (Positive Probiotic or Positive Prestarter _+ Probiotic), the number will be negative and on the left side of the zero. When the difference is statistically significant it will be above the red dotted line (significance cut-off or threshold).

INFECTIOUS CORYZA CLASSIFICATION, DIAGNOSTICS, AND A COMPREHENSIVE INVESTIGATION ON THE HMTP210 GENE OF *AVIBACTERIUM PARAGALLINARUM*

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SUMMARY

Infectious coryza is a worldwide respiratory disease of chickens caused by *Avibacterium paragallinarum*. Hemagglutination inhibition tests have been historically used to classify strains into serogroups A, B and C and serovars A-1 to A-4, B-1, and C-1 to C-4. Due to the difficulty in isolating the bacterium and the subjectiveness of serological results, the detection and classification of *A. paragallinarum* is moving towards a molecular-based approach. A few genotyping methods have been proposed using the HMTp210 gene. In this study, we thoroughly analyzed the HMTp210 gene with the goal of targeting regions of variability to attempt the correlation between genogroups and serogroups. Our 176 HMTp210 sequences were divided into four genogroups, I, II, III, and IV. Subsequently, we developed qPCR primers and probes for the detection of such genogroups that share high correlation to the already known serogroups. The full-length article will be published in a peer-reviewed journal to be determined.

INTRODUCTION

Infectious coryza is a bacterial respiratory disease that affects chickens worldwide. The disease is typically an issue in layer and breeder flocks that show a significant decrease in egg production, but respiratory disorders in broilers have also been reported, especially in the presence of concomitant agents (1).

Strains of the causative agent, *Avibacterium paragallinarum*, are serotyped based on their capability to agglutinate fixed chicken red blood cells (1). Hemagglutination inhibition (HI) tests were developed to classify strains into serogroups A, B and C (2,3), or serotypes A-1 to -4, B-1, and C-1 to –4 (4,5). However, the serotyping techniques for *A. paragallinarum* can sometimes result in subjective readings as cross-reactions may occur, and the array of sera used in the assays may affect the consistency of results in different labs. In addition, the HI assays are scarce worldwide, leading to expensive and hazardous shipping of live bacteria internationally.

To overcome issues seen with serotyping methods, we have thoroughly studied the HMTp210 gene, which is responsible for most of the hemagglutinating capability of *A. paragallinarum.* Our goal was to develop qPCR primers that can differentiate *A. paragallinarum* isolates into genogroups that are somewhat comparable to the current serogroups A, B, and C.

MATERIALS AND METHODS

A total of 176 full HMTp210 gene sequences of *A. paragallinarum* were used in this study. Of these, 100 (56.82%) were previously serotyped using either the Page or the Kume methods (3-5). All Kume serotypes (A-1 to -4, B-1, and C-1 to -4) were represented by at least one sequence.

A phylogenetic tree was constructed using the maximum likelihood method based on the GTRGAMMAI model with 1,000 bootstraps. Bootstrap values equal or greater than 70% and a nucleotide identity equal or greater than 95% were used as threshold to determine genotypes within the whole HMTp210 gene phylogenetic tree. Genotypes that shared ancestral nodes were considered to belong to the same genogroup.

Based on the phylogenetic results, four probe-based qPCR assays were designed to differentiate *A. paragallinarum* isolates into genogroups I (serogroup B), II (serogroup C), III (serogroup C), and IV (serogroup A) (Table 1). Seventy-five isolates stored in FTA cards that had already been genotyped were

processed for qPCR testing. Of the 75 specimens, 14 were typed as genogroup I, 6 as genogroup II, 20 as genogroup III, and 16 as genogroup IV. Nineteen samples were not previously genotyped.

RESULTS

The phylogenetic analysis of the whole HMTp210 gene of *A. paragallinarum* (Figure 1) showed that strains were divided into four genogroups: I, composed of mostly serogroup B isolates and is divided into four genotypes (1a to -d); II, with predominantly C-2 isolates from North America and one C-3 prototype from South Africa; III, with serotype C isolates from worldwide distribution; and IV, which bears serotype A strains and is divided in genotypes 4a, -b, and -c. The greatest within-genogroup diversity is seen in group IV. The qPCR assays developed based on the alignment of the 176 whole HMTp210 gene sequences were able to successfully differentiate between genogroups I, II, III, and IV.

DISCUSSION

Although serogroups and genogroups were consistent for the most part, some mismatches were observed. The hemagglutination capabilities of *A. paragallinarum* are mostly, but not exclusively, provided by the HMTp210 gene, and therefore, other bacterial genes might be playing a role in the current HI serotyping methods. In addition, the HI results for the isolates used in this experiment were performed in different laboratories worldwide. Cross-reactivity and subjectiveness in reading the HI results might have led to an inaccurate classification of some isolates. The former theory corroborates the demand to transition the classification of *A. paragallinarum* strains to molecular-based methods that are more consistent and easier to perform anywhere. The qPCR assays presented here are a step forward on the molecular screening of *A. paragallinarum* isolates for diagnostics and rapid classification.

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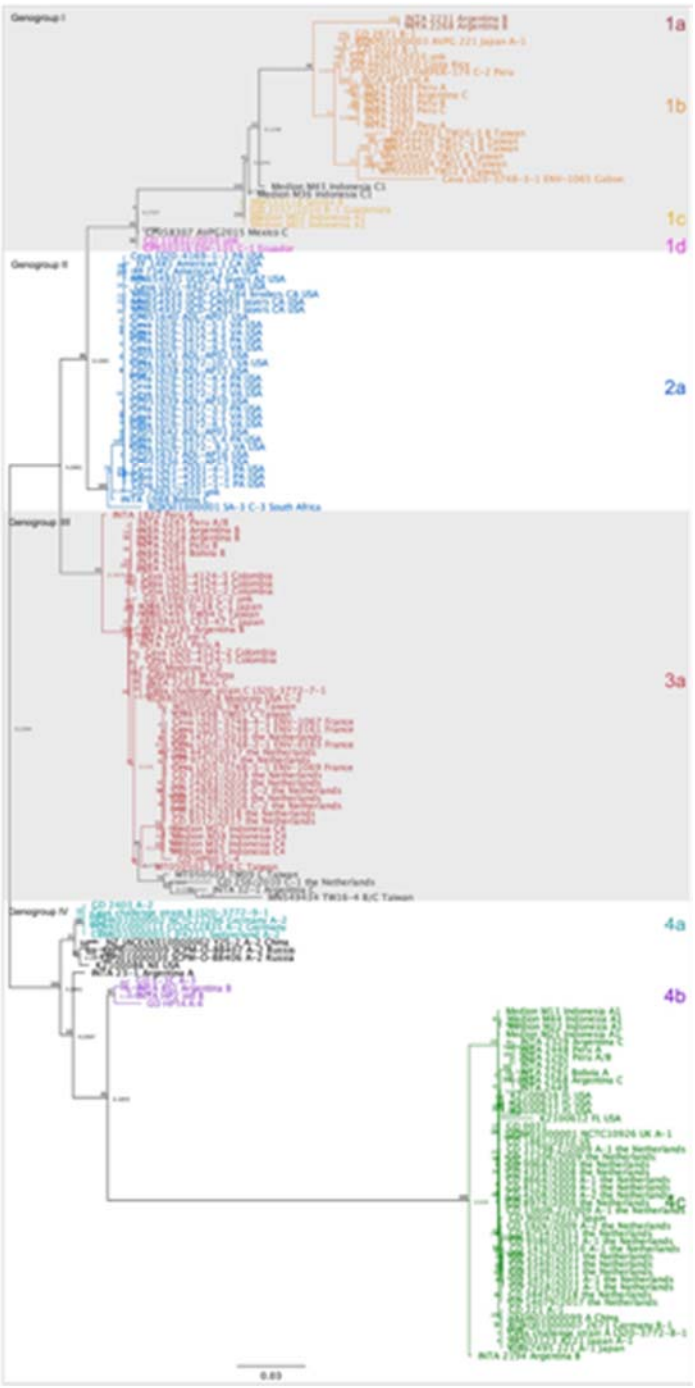
Table 1. Sequences or primer pairs and probes used in quantitative real-time polymerase chain reaction assays to differentiate *Avibacterium paragallinarum* isolates into Page serogroups A, B, and C.

Serogroup (genogroup)	Primers/ probes	Sequence (5'-3')	Fragment size (bp)
A (IV)	fAdS-A	GACMAATTGCCTGCTACTG	155
	rAdS-A	GCCTCCGGTTTATTAGGGTC	
	pAdS-A	FAM-GGCACTGTTCCGAAAAACTCC-BHQ	
B (I)	fAdS-B	GATTGTGGTTTCAGAGYTAG	205
	rAdS-B	CCTTAAGCATTTCAACACTTC	
	pAdS-B	FAM- GCGTCATTATTATTCTCACC-BHQ	
C1 (III)	fAdS-C1	GTCCCCTTTAGCAGCCAATACAATCGT	174
	rAdS-C1	GCATTCACCCCCATTGCTAATGAATCA	
	pAdS-C1	FAM- CAGGATCAAACAGTTTCGTAGGGGGTTC-BHQ	
C2 (II)	fAdS-C2	CGGTAGGTGAAGCGACAATTGC	88
	rAdS-C2	CCTAGTAATAAAGTCCCTGCTTGGCG	
	pAdS-C2	FAM- CAGATGTTGCAGCGGGGGCAC-BHQ	

Figure 1. Phylogenetic representation of 176 whole HMTp210 gene sequences of *Avibacteriumparagallinarum*. Sequences were divided in four genogroups and nine genotypes, each represented by a different color, based on nucleotide identities and bootstrapping values.



CASE REPORT OF COCHLOSOMIASIS IN TURKEYS

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SUMMARY

Cochlosomiasis is caused by a flagellated protozoan parasite, *Cochlosoma anatis*. *C. anatis* causes enteritis and severe morbidity affecting the growth rate of turkeys. In February 2023, enteritis was noticed in a single-age grow-out Antibiotic Free (ABF) turkey farm in the Southeastern United States. Wet mount examination revealed *C. anatis*, which was consistent with the PCR and histopathology results. In addition, *Eimeria* oocysts were identified on microscopic examination and confirmed as *E. meleagrimitis* by PCR, but no histopathological changes associated with *Eimeria* were noticed in the intestine.

INTRODUCTION

Flagellated protozoa affecting the intestinal health of turkeys include *Histomonas*, *Cochlosoma*, *Hexamita*, *Trichomonas*, *Tetratrichomonas*, and *Pentatrichomonas* (1,2,3). *C. anatis*, a flagellated protozoan parasite, is often associated with enteritis in turkeys. It affects the growth performance of turkeys, inducing high morbidity, lethargy, and affects the uniformity of the flock. Young turkeys are severely affected with cochlosomiasis. Neither prophylactic/therapeutic treatments nor vaccines are available to prevent *C. anatis* infection. *C. anatis* viability outside of the host is minimal, and thus the transmission between the flocks can be controlled by adhering to strict biosecurity measures.

CASE HISTORY

In February 2023, enteritis was noticed in two houses on a single-age grow-out Antibiotic Free (ABF) turkey farm (n=5096/house) at 40 days-of-age located in the Southeastern United States. Necropsy was performed and intestinal samples were collected.

MATERIALS AND METHODS

Wet mount examination. Intestinal mucosal scraping was collected and visualized under a microscope.

Histopathology. Intestinal samples were collected in 10% neutral buffered formalin for histologic examination. Intestinal samples were paraffin embedded and sections were stained with hematoxylin and eosin following standard histologic procedures. The stained slides of intestinal sections were evaluated by following standard histologic procedures.

Molecular diagnostics. DNA extraction was performed from pooled intestinal samples by using DNeasy® Blood & Tissue Kit (Qiagen) by following manufacturer's instructions. DNA was extracted and PCR was performed targeting mitochondrial cytochrome *c* oxidase subunit I (mtCOI) locus of turkey *Eimeria* species (4) and 16S rRNA gene of *C. anatis* (5). The amplicons generated from the PCR were purified and submitted for sequencing.

RESULTS

Wet mount examination. *C. anatis* was identified based on the morphology and characteristic motility. In addition, *Eimeria* oocysts were also detected on microscopic examination.

Gross pathology. The intestines were pale and lacked the normal tone. White mucoid contents were noticed in the small intestine.

Histopathology. Duodenum had mild to marked atrophy of the villi with dense colonization of the tips and sides of the villi with myriad protozoal organisms (*Cochlosoma*) attached to the mucosal surface and free in the lumen. Jejunum had chronic enteritis characterized by shortened villi. In the small intestine, bacterial rods of mixed morphology were mixed with mucus on the tips and sides of the villi. Cecum had subacute to chronic protozoal typhilitis with colonization of myriad protozoal organisms (*Cochlosoma*) on the mucosal surface and the sides of the crypts. Histologic evidence of coccidiosis was not identified at any level of the intestine sections.

Molecular diagnostics. PCR performed against mitochondrial cytochrome c oxidase subunit I (mtCOI) locus of turkey *Eimeria* species had a positive band for *E. meleagrimitis* and was confirmed by sequencing. PCR performed against 16S rRNA

gene of *C. anatis* had a positive band and was confirmed by sequencing.

DISCUSSION

Turkey protozoal enteritis associated with flagellated protozoa has been sporadically documented worldwide. *C. anatis* is one of the flagellated protozoa causing significant losses due to enteritis, stunting and by affecting the flock uniformity. Microscopic examination of the intestinal mucosal wet smear revealed the presence of *C. anatis* and was supported by PCR. Histological evaluation of multiple levels of small intestine and ceca revealed villi injury with myriad protozoal organisms, consistent in size and morphology with *Cochlosoma*. In addition, bacterial rods were mixed with mucus on the tips and sides of the villi, representing dysbacteriosis. The compromised intestinal integrity provided an opportunity for secondary bacteria to evade the intestinal layer leading to dysbacteriosis. In addition, *Eimeria* oocysts were detected during microscopic examination of the intestinal mucosal wet smear and confirmed as *E. meleagrimitis* by PCR and sequencing, but no histologic evidence of coccidiosis was identified in the submitted intestinal samples. With the unavailability of prophylactic/therapeutic measures, management procedures such as focusing on biosecurity and sanitation minimizes the spread of cochlosomiasis between turkey farms.

ACKNOWLEDGEMENT

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OTHER CECAL WORMS IN COMMERCIAL CHICKENS

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ABSTRACT

Heterakis gallinarum is a very prevalent poultry parasite found in the ceca of various species of gallinaceous birds. *H. gallinarum* recognized as an economically important parasite due to its role as an intermediate host for the protozoal parasite *Histomonas meleagridis* which causes histomonosis. In recent years, there have been mortalities in broiler breeders and litter raised commercial egg production chickens in the Eastern United States. Some cases were investigated, and the gross lesions were necrotic patches in the ileum and nodular granulomas in the ceca. Microscopic examination of cecal scraping confirmed He*terakis* spp. Further evaluations showed that *H. isolonche* was also a portion of the *Heterakis* family. Another helminth member of the cecal population was the *Subulura brumpti*. *H. isolonchi* and *S. brumpti* are two species reported to cause mortality in some avian species, pheasants, and other game birds, but not broiler breeders.

INTRODUCTION

During recent years, the poultry industry has seen moderate growth; broilers averaged 67 percent of all poultry sales, chicken eggs designated for human consumption averaged 22 percent, and turkeys averaged 11 percent of all poultry sales. Each fraction of the poultry sector has young, developing, and mature birds. The environmental conditions may differ for these animals during different periods of their production periods. Other factors that have impacted poultry production practice are consumer's demands and or legal requirements. There is a growing interest in having the production animals living in less confined spaces as compared to earlier years of our industries. In recent times, some egg-laying birds are raised in houses with litter floors and free-range systems. Also, for the meat-type birds the young animals may start on previously used bedding as compared to years ago when new bedding /litter was the requirement. Egg-laying birds will be in these environments for rather long periods and will therefore have a greater chance of being exposed to foreign organisms such as helminths.

On occasions, flocks of broiler breeders and commercial egg laying operations in the Eastern United States would have unexplained mortalities. *Heterakis* spp. are common poultry parasite; these parasites inhabit the ceca of several species of gallinaceous birds (1,2,3,6). *H. gallinarum* is of economic important due to its role as an intermediate host for the protozoal parasite *Histomonas meleagridis. H. meleagridis* causes histomonosis in several avian types. *H. isolonche* has also been reported to parasitize avian species such as pheasants and may be associated with morbidity and mortality (1,2). There are other cecal helminths (*Subulura* spp.) that have been reported to parasitize the avian host and suspected to be associated with clinical signs (5,6).

MATERIALS AND METHODS

During the past three years, over 300 accessions were conducted on intestinal samples from different types of poultry. Some of the samples were from farms that may have had morbidity and or mortality; several of these cases were treated in accordance with the initial diagnosis. However, in some of the cases the animals did not respond to the prescribed medication and therefore, a second opinion was sought as to the cause of the mortality.

Wet mount preparations. Wet mount smears were prepared from the duodenum, jejunum, ileum, ceca and rectum. Smears were prepared by using a drop buffered saline on a clean microscope slide, content added then examined at 100x and 400x magnifications. All parasites or parasitic stages were document.

RESULTS AND DISCUSSION

The most prevalent enteric parasites were helminths followed by *Eimeria*. Cecal worms were the most common followed by *Ascaradia* spp., then *Capillaria* spp., and cestodes the least prevalent. A report from Pakistan reported similar findings, that *H. gallinarum* was the more prevalent than *A. galli*, (6). But these samples were from backyard sources. Another paper from India, demonstrated that birds grown in free-range environments harbored eight

types of worms (four types of nematodes and four types of cestodes); the most prevalent were *A. galli* 29.6%, *H. gallinarum* 24% from the nematodes and *R. cesticillus* 19.2% and *R. echinobotheridia* 13.2% from the cestodes, (3). The findings reported here showed that most of the enteric parasites were nematodes 50%, followed by cecal worms 36% and cestodes 5%, respectively, Table 1.

The pattern from this report follows a similar trend as the previous reports, but those reports were from birds in backyard or free-range environments. The data in this report were from commercial broiler breeders and commercial eggs farms. The breakout of the types of cecal worm infestations were *H. gallinarum* 43%, followed by *H. isolonche* at 22% and *Subulura brumpti* at 13%. Other authors that have reposted on *H. isolonche* in game birds were (1,2) in USA and Brazil, respectively. But the current findings are from commercially grown chickens. Reports on the *Subulura brumpti* game birds and none commercially grown chickens (4,5). This article documents the findings of these unique cecal parasites from commercially grown chickens.

SUMMARY/CONCLUSION

Heterakis spp. are inhabitants of the ceca and are particularly important poultry parasites due to the being the intermediate host for a very devastating protozoan diseases histomoniasis cause by *Histomonas meleagridis.* This investigation revealed that just identifying cecal worms as the agent may not be good enough. Efforts should be made to determine the genus and species of these agents. These two unique organisms (*H. isolonchi and S. brompti*) have shown to be associated with morbidity and mortality in poultry and have been identified in our commercial poultry populations.

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Table 1. The types of enteric parasite encountered.

Enteric parasites found in commercial chickens				
Eimeria	*acervulina, brunetti, maxima, mivati, praecox, hagani, necatrix, tenella, mitis*			
Helminths	*Ascaridia galli*	*Capillaria obsignata*	*Heterakis*	Other spp.
			gallinarum	*Subulura brompti*
			isolonche	*Strongyloides* spp.
Cestodes	*Choanotaenia infundibulum*	*Raillietina cesticellus*		

NEW INFECTIOUS BRONCHITIS INDIRECT ELISA, BASED ON WELL CONSERVED RECOMBINANT PROTEIN, FOR IMPROVED DETECTION OF LIVE VACCINES AND CHALLENGE INCLUDING VARIANT STRAINS

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SUMMARY / INTRODUCTION

Avian infectious bronchitis virus (IBV) is a coronavirus, which infects poultry and causes infectious bronchitis. It is a highly infectious avian pathogen, which affects the respiratory tract, gut, kidney and reproductive systems of chickens. Important mutations could be observed for avian coronavirus which lead to the appearance of new variants worldwide. To control the disease, vaccination is largely used and based on classic or circulating variant strains. Thus, diagnosis and monitoring of vaccination require laboratory testing, and ELISA may be used for monitoring serum antibody responses. The new ID Screen® Infectious Bronchitis Indirect ELISA (IBVARSV2), based on well conserved recombinant protein, allows for the detection of IBV antibodies in samples. This kit is specifically used for the detection of antibody response after IBV vaccination (including variants: 4/91, 793B, QX, It02, Var02 BR1…), and improve detection of challenge in vaccinated flocks (by classic or variants strains). A dedicated baseline was established with this IBV iELISA to monitor vaccination in the field. The objective of this paper is to summarize performances obtained with this new IBV indirect ELISA, based on highly conserved protein.

MATERIALS AND METHODS

Diagnostic specificity. The diagnostic specificity represents the percentage of samples belonging to negative population, identified as negative. 172 SPF chicken sera from France (INRA-Tours) were tested using the ID Screen Infectious Bronchitis Indirect ELISA.

Inclusivity and exclusivity. Inclusivity and exclusivity were evaluated by the testing of specific monovalent samples. A panel of samples obtained after inoculation of chickens with classic or variant strains of the Infectious Bronchitis virus, and a panel of serum samples collected from diseased chickens, were tested with the ID Screen Infectious Bronchitis Indirect ELISA. This panel was provided by GD Animal Health (Deventer, The Netherlands).

Analytical sensitivity. Analytical sensitivity was evaluated through the titration of reference sera from chickens infected with IBV classic or variant strains (GD Animal Health, Deventer, The Netherlands). Sera were tested in parallel on the ID Screen Infectious Bronchitis Indirect ELISA, and two others commercial ELISAs.

Diagnostic sensitivity. The diagnostic sensitivity represents the percentage of samples belonging to positive population, identified as positive. The sensitivity of the ID Screen Infectious Bronchitis Indirect ELISA was evaluated with a set of samples, vaccinated with different commercial vaccines. The mean titers, CV% and percentage of positivity was evaluated for each study.

Vaccination with one live vaccine was evaluated in the study #1. The vaccine used was the Poulvac® IB Primer (Zoetis Schweiz GmbH), a live vaccine containing both classic H120 and variant D274 strains of IBV. 9 broiler flocks (origin: Czech Republic) were vaccinated at one day of age with the Poulvac IB Primer, by spray, and no further dose was applied before they were bled at slaughterhouse, when they were between 34 and 37 days of age.

Then, vaccination with 2 mass live and 1 variant live vaccine was evaluated in the study #2. 7 broiler flocks (origin: Malaysia) were vaccinated with this vaccination program and bled at slaughterhouse.

Finally, the study #3 was performed to evaluate vaccination with 1 killed vaccine plus boost within different flocks: 11 breeder layer flocks (origin: Malaysia), bled between five and eight weeks post-vaccination (wpv) [study#3a]; and 2 layer flocks (origin: Jordan), vaccinated with 1 inactivated vaccine (mass + 4/91) + live vaccines (H120 or 4/91), and bled at 17 and 26 weeks of age [study#3b].

RESULTS

Diagnostic specificity. 172/172 sera were found negative with the ID Screen ELISA. The measured

specificity with the IBV iELISA is 100 % ($CI_{95\%}$ [97.82, 100.0], n=172).

Inclusivity and exclusivity. All the following IBV strains were found positive with the IBV iELISA: Beaudette, D274, D1466, D3128, 4/91 (793B), M41, D8880, D388 (QX) and Italy 02. The IBV iELISA allows high detection of variants. All sera belonging to other avian pathogens were found negative the IBV iELISA.

Analytical sensitivity. The ID Screen ELISA is able to detect a low amount of antibodies within weak positive samples. It offers a high analytical sensitivity, with good detection of both classic and variant IBV strains, in comparison to the other commercial ELISAs. High analytical sensitivity can notably allow early detection of challenge by a field IBV strain.

Diagnostic sensitivity.

Study #1: for flocks vaccinated with one live vaccine, the expected antibody titers are between 4000-8000 (indicated as the baselines). Among the 9 vaccinated flocks, 2 were identified as well vaccinated, with titers including in the baseline. Mean titers obtained was 4841 and 5320, with CV% around 70 and percentage of positivity over 80%. At the opposite, challenge was identified in the 7 remaining flocks, with high antibody titers (> 10000), or high CV% associated with important amount of negative samples.

The IBV iELISA is a high-performance tool for the monitoring of live vaccine.

Study #2: for broiler flocks vaccinated with 2 Mass Live and 1 Variant Live vaccines, the expected antibody titers are between: 6000-9000 (indicated as the baselines). With mean antibody titers between 5496 and 8127, flocks 1, 2, 3, 4 and 6 are within the baselines, in agreement with a good vaccination application. With mean antibody titers higher than the baseline (superior to 10 000) and reduced CV%, a challenge is suspected in flocks 5 and 7.

Study #3: for non-challenged broiler flocks vaccinated with 1 Mass Killed plus Multiple Live (classic and variant) vaccines, the expected antibody titers are between: 8000 – 13000 (indicated as the baselines). For challenged flocks vaccinated, the expected antibody titers are > 13000.

Study #3a: with mean antibody titers between 8303 and 12612, 6 of the 11 tested flocks are within the baselines, in agreement with a good vaccination application. For the last 5 tested flocks, antibody titers obtained were close or superior to 13000, with low CV% values. A challenge has been suspected. This was confirmed by further investigation by PCR (data not shown), with the detection of QX strains.

Study #3b: for flock 1, the antibody titers obtained were between 8000 – 13000 with maximum titers below 17000, within the baselines, in agreement with a good vaccination application. For flock 2, the antibody titers obtained were close to the baselines, but with a maximum titer superior to 17000, suggesting an IBV challenge. Consequently, flock status was further investigated by RT-qPCR test targeting IBV variant 02. Samples collected at 26 weeks of age, were found positive for this variant strain. It has to be noted that the IBV variant 02 was not a strain included in the vaccination program applied to this flock, confirming challenge by a field IBV strain.

Study #2 and #3 shows high and better detection of challenge with variants strains under vaccination.

CONCLUSION

The ID Screen Infectious Bronchitis Indirect ELISA, based on a well conserved recombinant protein demonstrates high analytical specificity, without cross-reaction with other avian pathogens. It shows an improved detection of all existing IBV variants (including 4/91, 793B, QX, It02, var02…), with high analytical sensitivity compared to other commercial ELISA. It is a good tool for the monitoring of vaccinated population and for the detection of antibody responses to live and attenuated vaccines (classic or variant IBV strains), and the identification of challenges (including by IBV variants) in vaccinated animals. Accurate baselines are required for serology interpretation.

AVIAN HEPATITIS E VIRUS CASES IN LAYING HENS IN THE U.S.

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ABSTRACT

Poultry flocks starting at 40 weeks of age in different states in the U.S. have been dealing with clinical signs, mortality, and economic losses that resemble avian hepatitis E virus infections. We have screened several flocks for the presence of antibodies finding a seroprevalence of 23%. In addition, we worked on a RT-qPCR to detect the presence of the virus in liver, spleen, gallbladder, and bile. Upon detection of the virus in organs, we started the isolation process in embryonated SPF eggs via IV and LMH cells. Since this virus shows a fastidious growth behavior in conventional virological cells, in the meantime, we worked on getting the full genome sequence of two aHEV viruses from bile and liver. The results of this sequencing effort ended in the detection of genotype 2 which was previously reported in the U.S. and genotype 3, new to the country. We worked on fulfilling Koch postulates.

INTRODUCTION

Avian hepatitis E virus (aHEV) is a non-enveloped, single-stranded RNA virus in the genus *Orthohepevirus* (1). This virus induces the hepatitis-splenomegaly syndrome (HSS) (2) affecting broilers, layers, and breeders. In layers, the syndrome can induce high mortality rates and subsequent decreased egg production. (3) The virus has also been detected in birds without HSS clinical signs (4). The diagnosis of HEV is complicated due to the lack of an efficient virus recovery and amplification system, complicating subsequent identification and characterization of the pathogen. While studies report the isolation of the virus in eggs or cells, methodologies are complicated, and yields are very low (5,6). Since virus isolation is not always successful, diagnostics have been based on serology, specifically enzyme linked immunosorbent assay (ELISA) tests. Reverse transcription polymerase chain reaction (RT-PCR) is the most effective technique to detect the avian HEV genome. Among these different molecular techniques SYBR Green RT-qPCR is characterized by its simplicity, low cost, fast detection times and high sensitivity and specificity. However, despite all those benefits no validated test is available for the detection and quantification of aHEV in the United States.

The objectives of the present study are to: (a) Determine the seroprevalence of the disease by detecting the presence of antibodies against aHEV in layer farms in the U.S. and establish patterns of seroconversion, (b) develop and test a SYBR Green RT-qPCR for the identification and quantification of aHEV RNA in liver, spleen, gallbladder and bile, in natural infections (c) identify the aHEV strains circulating in the U.S. layer chicken population, (d) attempt the isolation aHEV strains from field samples and upon obtention of a virus isolate test Koch postulates in pullets.

MATERIALS AND METHODS

Sample collection. Blood, liver, spleen, gallbladder, and bile samples from egg layers between 30 and 50 weeks of age, showing clinical signs of illness were submitted to the UC Davis Poultry Medicine laboratory for diagnostic work and further testing.

Electron microscopy. Seven liver and three spleen samples were processed for negative contrast electron microscopy following previously described methodology (7).

aHEV antibody ELISA. Serum samples were evaluated for aHEV antibodies using the BLS ELISA kit (BioChek, UK, Ltd.) per the manufacturer's guidelines.

SYBR-Green RT-qPCR. ORF 3 was analyzed in several genomes obtained in GenBank to construct primers F1: 5-'GCTCATGCTTGCAATGTGCT-3' covering nucleotides 4745-4764 and R1: 5-TCGTAACGTTCGTACTCG-3' covering nucleotides 4908-4925. A series of 10-fold dilutions of standard gBlocks were prepared. The dilutions ranged from 10^{10} to 10^{2} copies per microliter in DEPC water. An RT-qPCR from each dilution was performed in triplicate. The Ct values obtained were plotted against the corresponding DNA copy numbers to construct a standard curve.

Nanopore sequencing and phylogeny. Positive samples from the RT-qPCR were used for whole genome sequencing. The genome was amplified using two primers F1: 5-'TGGACGTCTAGTTTGCAGAGTCCA-3' covers nucleotides 31-52 (EF206691), and the R1: 5-ACACTGCCCGAAATGGGAGGATTTC-3' covers nucleotides 6641-6665 (EF206691). Products were purified and sequenced using standard Nanopore protocols. A multiple sequence alignment of the entire sequences was created in Geneious Prime 2020.1.1 using the MAFFT plugin. The maximum-likelihood method was employed to build phylogenetic trees based on the GTRGAMMAI model, with 1000 bootstrap replicates in Geneious Prime, utilizing the RaxML plugin.

Virus propagation in SPF eggs and LMH cells. Samples were prepared by maceration and filtration under standard virus isolation protocols. Embryos were inoculated intravenously in 11-day-old SPF eggs according to Clavijo *et al*., (8). Incubation for nine days post-inoculation was followed by candling to identify viable embryos. At 9 days post-inoculation, viable eggs were refrigerated, and controlled cracks were made for embryo extraction. Tissues of liver, gallbladder and intestines were carefully collected and used as the inoculum for the next passage. The harvested tissue was assessed for viral quantification using the previously described RT-qPCR. In parallel, LMH cells were cultured in DMEM medium supplemented with 10% FBS at 37°C in a 5% CO2 environment. We worked with three groups: aHEV positive (livers), negative control, and mock control (PBS). Liver samples were homogenized at 10% in sterile PBS, centrifuged, and filtered before inoculating the LMH cells for two hours at 37°C. After eliminating non-adsorbed virus using PBS, the medium was replaced with low-serum medium (10% FBS/DMEM). Half of the culture was frozen for RT-qPCR, while the other half was left for replication. After 48-72 hours, supernatants and cells were collected.

RESULTS AND DISCUSSION

Only one liver sample was encountered to be positive. At the EM the viral particles showed an icosahedral symmetry and lacked an envelope. Their diameter was between 30 and 33nm (Figure 1). The finding of positive livers is not surprising since the virus after replicating in the intestines does viremia migrating to the liver and spleen, where it concentrates. Antibodies anti-aHEV were detected in 64 out of 210 serum samples, giving a seroprevalence of 30%. Different prevalence was observed depending on the age of the flock. A seroprevalence of 18.4% was seen between 20 and 50 weeks of age (7/38); 38.8% (49/126) between 51 and 80 weeks, and finally 17.4% (8/46) between 81 and 110 weeks (Figure 2). This might be associated with the infection dynamics of aHEV. While the birds start getting infected in the early stages of production, there is a peak of infection between week 50 and 80 declining after 80 weeks. These serology results agree with the clinical picture occurring around week 30-50 declining as the birds age.

The virus was detected by RT-qPCR in all studied tissues. A significantly higher viral load was detected in gallbladder samples compared to livers and spleen samples. Spleen samples showed significantly lower viral load compared to the other tested tissues. (Figure 3).

The higher virus accumulation in the gallbladder might be due to the replication occurring in the liver and concentration in the bile fluid. This accumulation allows the use of the bile as an inoculum since it is a very easy sample to work with.

Sequences were obtained from four isolates. Two of these isolates; were identified as genotype 3 with an identity percentage ranging between 83 and 94%. The other two were classified within genotype 2. Their identity percentage with reference sequences was between 88.2 and 90.4%. (Figure 4). While genotype 2 was previously reported in the U.S. (9) genotype 3, is new to the country. This last detection suggests a potential introduction to the country through vertical transmission since this genotype is similar to a virus sequence uploaded in GenBank from an aHEV isolated from Hungary uploaded in 2005.

As far as virus isolation the IV egg inoculation did not yield the expected results. Both samples of bile and liver reduced the viral load after the first passage and became negative after the second passage. Surprisingly, passages in LMH cells yielded better results. After one passage the samples showed a reduction in viral load, this reduction was sustained after the second passage. After the third and fourth passage we detected an increased load in the samples. While the viral load values are not extremely high this increase is an indication of effective replication. These results suggest that there might be a line or cell susceptibility that makes this virus less prone to replicate in certain culture systems.

In conclusion, this study contributes with valuable information on seroprevalence, diagnostics, identification of new genotypes, and the insights into viral isolation challenges. Further research is required to enhance our understanding of aHEV in commercial layers in the United States. Results of the Koch postulates fulfilment will be shared at the meeting.

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(An extended version of this manuscript will be published in a journal to be determined.)

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Figure 1. Transmission electron microscopy of liver samples from aHEV positive tissues by RT-qPCR. Two viral particles can be noticed. They show capsids with icosahedral symmetry and lack of envelope.

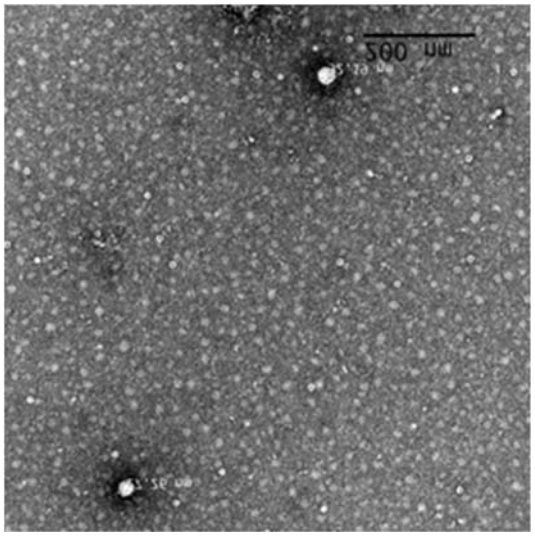


Figure 2. Antibody titers to Avian Hepatitis E virus on serum samples from layers in production. Higher titers are observed between weeks 51 and 80 of age; however, no significant differences were detected.

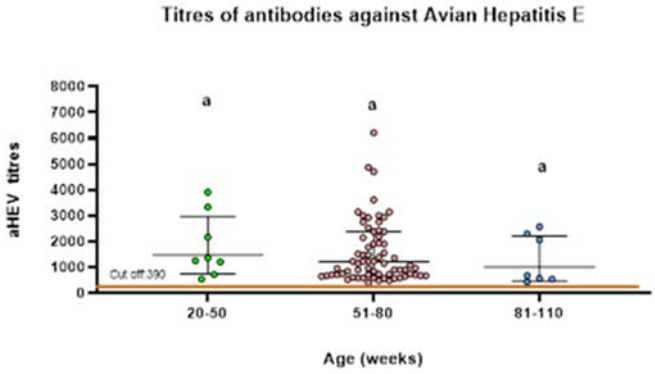


Figure 3. HEV viral load was determined using RT-qPCR. The difference between viral loads between groups was statistically significant.

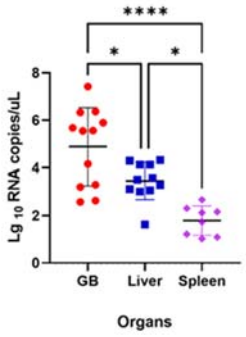
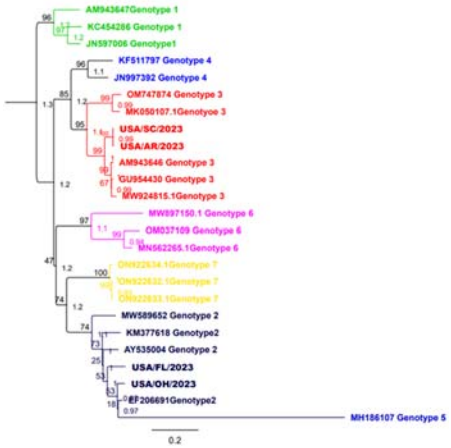


Figure 4. Phylogenetic tree derived from the complete genomic nucleotide sequences of Avian Hepatitis E. This analysis involved 25 nucleic acid sequences. All genome sequences obtained from GenBank are labelled with the name, accession number in parenthesis, and the designation of the corresponding genotype. Sequences obtained in the present study are labelled in bold. Samples analyzed for this project group with genotype 3 and genotype 2 sequences.



AVIAN REOVIRUS VARIANTS, APPLIED VERSUS THEORETICAL RESEARCH

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Since 2011 an outbreak of avian reovirus (ARV) variants has been affecting the poultry industry in the US and the world (1, 2). While reoviruses in poultry have been associated with respiratory, digestive, poor performance, immunosuppression, and locomotory disorders, this current outbreak has been characterized in meat type chickens by arthritis tenosynovitis, lameness and consecutive locomotory and welfare issues. This is not a new disease; the virus was first isolated from tenosynovitis cases by Olson in 1955 (3) and characterized by Walker in 1972 (4). The main issue is that, this time around, is caused by avian reovirus variants that escape from the conventional vaccines elicited immunity. This is the main reason why the current vaccination strategy doesn't work. Despite heavy vaccination of breeding hens, offspring are not fully protected, possibly due to the many variants with broad antigenic diversity. Avian reoviruses are highly variable viruses its genome conformation allows them to evolve rapidly, reason why there have been up to seven genotypes described (1, 2, 5).

Three key points are important to control endemic variable viruses, causing disease in commercial poultry:

1. Identification
2. Surveillance
3. Effective control and prevention strategy (Vaccine, management biosecurity, C&D)

Using this strategy, you hope to see a reduction in cases, a reduction of viral load per case (which allows reduction of the challenge in the environment) and increase age at case submissions to the diagnostic laboratory.

Diagnostics and identification. Clinical signs caused by ARV variants are usually related with lameness, poor performance, increase in condemnations in broilers and uniformity issues. At gross pathology you can see swollen joints, edema of the gastrocnemius and digital flexor tendons and necrotic foci on the epicardium (6). At microscopic pathology it can be noticed tenosynovitis with lymphoid infiltration. This lymphoid infiltration can be also seen in the myo- and pericardium (6, 7). In addition, you can notice lymphoid depletion in the thymus and bursa of challenged SPF chickens (7).

In terms of sampling, the best is to send sample to the laboratory, so tissues can be collected aseptically. Recommended samples are tendons and sheaths, heart, and its pericardium. The virus is isolated in chicken embryo liver cells (CEL's), this step is important because it will provide enough genetic material for molecular characterization. In addition, allows further characterization. Reovirus indices a cytopathic effect in CEL's forming synctitia. Alternatively, SPF embryonated eggs can be used, ARV's will cause mortality and hemorrhagic lesions in infected embryos. Alternatively, whole legs can be sent to the laboratory with heart + pericardial sac. FTA cards and RNA/DNA Shield® has been used, with relative success, to ship samples to other countries with the adequate permits. Usually RT-qPCR is used to determine the presence of the reovirus. In addition, clinical signs and histopathology validate the molecular detection with pathological changes associated with ARV.

Surveillance. Surveillance is extremely important to strategize control and prevention measures against ARV. Variant detection changes the control and prevention strategy since cross protection is not always elicited by conventional vaccine strains (Derived from S1133). While the fastest and easiest way of surveillance is through molecular characterization, using a segment of ~1,088bp of the S1 gene this is not fully informative. It will allow epidemiological understanding but will only provide partial antigenic information. That is why is always better to pair molecular surveillance with antigenic and pathological characterizations. The practicality of the last two is debatable.

Regarding molecular characterization, 6 (1, 2) or 7 genotypes (5) have been described based on the sequence of a portion of the S1 gene (8). This information needs to be analyzed carefully because a proportion of isolates could have combined strains of reovirus.

Control and strategy. The control and prevention strategy will depend on the presence or absence of variant strains. Commercial vaccines using

conventional ARV strains are available in the market. These can be live and killed products. The presence of variant challenges will require the use of antigenically relevant strains in the vaccination program since poor cross protection has been seen between viruses on different genotypes and sometimes inside the same genotype. The strategy is based on hyperimmunization of breeders that provide relevant maternal antibodies to the progeny to push the challenge for at least the first two weeks of life of the broiler. These urges the need of Autogenous vaccines that need to match the challenge in the field. This strategy should be followed by evaluation of takes after vaccination in breeders and evaluation of the maternal antibodies delivered to broilers. In addition, a reduction of the challenge in the field through biosecurity, cleaning and disinfection and proper down time between flocks is a must. Through this, the challenges are pushed to later in the chicken's life taking advantage that chickens become refractory to the ARV infection as they age. In addition, management problems reduction to diminish predisposition to infections, control of immunosuppressive diseases (that reduce events that generate variant strains) are important to consider.

While using this strategy sometimes provide promising results, we are not solving the main issue, doing this requires basic understanding of ARV's antigenic determinants. Basic efforts to understand this have been carried out by our laboratory. We have found that variant strains are genetically and pathologically different than conventional strains (7). We have also found that besides S1 the L3 and M2 genome segments are highly variable and might be related with antigenicity (7). Finally, we have worked on antigenic cartography which is a technique that uses functional data i.e. cross neutralization indexes to associate antigenicity between strains (Figure 1). As seen in figure 1, each square represents an antigenic unit (AU) and is equivalent to a 2-fold dilution in antibody titer. Using statistics, we calculated functional (antigenic) clustering, these clusters are represented by different colors.

In summary, control of endemic RNA pathogens is very difficult due to its inherent variability. Multiple variants of ARV have been described. It is important to proper diagnose ARV in poultry, for that, a combination of molecular techniques, virus isolation, history, and associated pathology is needed. Surveillance provides important epidemiological information and while the antigenic information is incomplete it is necessary for establishing control and prevention strategies. Finally, a good control strategy should involve hyperimmunization of breeder flocks using relevant strains that match the strains challenging birds in the field plus management, cleaning and disinfection and biosecurity to reduce the challenge in the field. This strategy should be improved by understanding of the antigenic and pathogenic determinants of these viruses through basic research.

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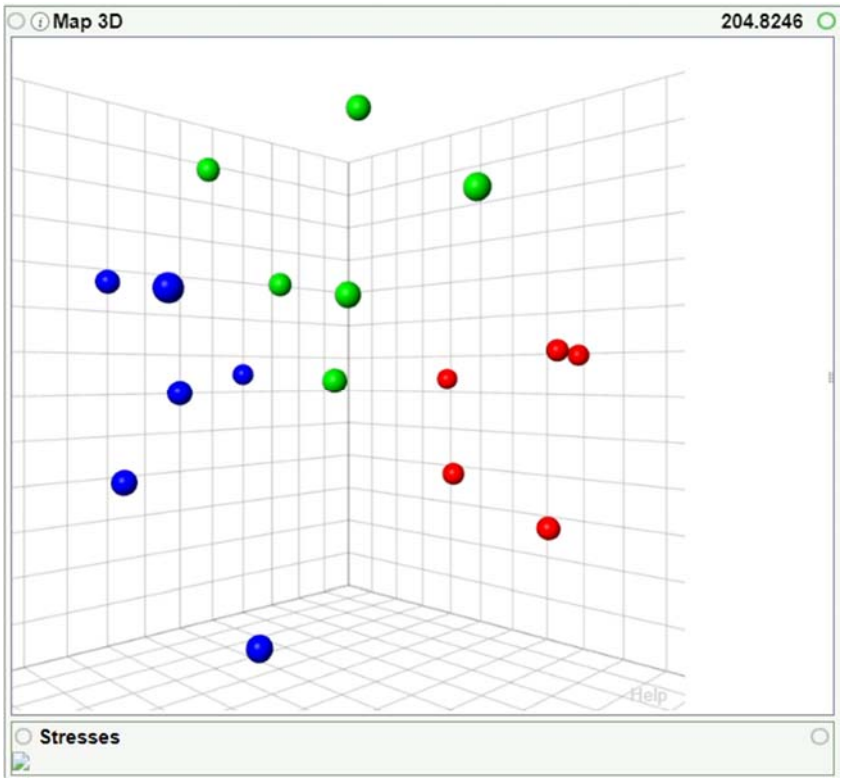
Figure 1. Antigenic cartography of 17 ARV viruses. VN indexes were plotted in a graph in which each square represents 1 antigenic unit, which is the equivalent to two-fold dilutions. The different colors represent different antigenic groups.



AN ANALYSIS OF NECROPSY CASES OF SALT TOXICITY DIAGNOSED IN CHICKENS IN CALIFORNIA: 2014–2023

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INTRODUCTION

Salt toxicity (ST) in confinement reared poultry has been intermittently reported and can lead to economic losses to the poultry industry (1). ST can be induced by excess intake of sodium from feed and/or drinking water or directly from restricted water intake. ST compatible gross lesions in chickens and turkeys include generalized subcutaneous edema, brain edema, ascites, hydropericardium and cardiomegaly, edematous and congested lungs, pale and edematous kidneys, and enlarged and cystic testes (1–7). An age susceptibility to ST has been reported in young, intoxicated birds (7). The aim of this study was to characterize the clinical, pathologic and toxicologic findings in a case series in chickens diagnosed with ST at the California Animal Health & Food Safety Laboratory System (CAHFS) from 2014 to 2023.

MATERIALS AND METHODS

The archives of the CAHFS laboratories were searched for chicken necropsy cases with a diagnosis of ST from January 2014 to July 2023. A total of 66 chickens from seven commercial and backyard chicken cases were retrieved, and analyzed. Live chickens were euthanized by CO_2 gas. Full postmortem examination and diagnostic work-up were carried out in all birds.

Samples of the skin, nasal cavity and sinus walls, eye, eyelid, cerebrum, cerebellum, heart, trachea, lung, liver, kidney, adrenal glands, testis, ovary, spleen, kidneys, air sac, yolk sac, navel, pancreas, intestines, and bone marrow were collected and fixed by immersion in 10% buffered formalin (pH 7.2) for 24–48h. All tissues were processed by standard histological techniques to produce 4-µm-thick hematoxylin & eosin (H&E)-stained sections. Twenty-four brains from live and dead chickens were bagged individually and submitted for sodium analysis. Feed samples submitted from 5 cases were also analyzed to determine sodium levels.

RESULTS

ST compatible gross findings were described in most of the seven cases considering ascites, anasarca, hydropericardium/cardiomegaly, edematous congested lungs, swollen testis, and pale enlarged kidneys. Other gross findings described were distended, thin-walled intestines with watery content, and distended large intestine with a diffuse, diphtheritic pseudomembrane on the mucosa.

H&E stained slides revealed ST compatible microscopic findings were noted in most of organs of all seven cases including edema in brain, heart, lung and testis. Brain sodium levels were less than 1,800 ppm in 19/24 brains tested, whereas brain sodium levels were equal to or higher than 1,800 ppm in 5/24 cerebral samples. Elevated levels of sodium were detected in 5/7 feed samples analyzed.

DISCUSSION

A case series of natural ST affecting broiler and backyard chickens over a period of nine years was analyzed. Compatible ST gross and microscopic lesions were described from most cases and lesions were consistent with previously reported ST avian cases (1–7). Cystic testis, previously identified as a sequel of ST in chickens (4), was grossly and microscopically described in 6/7 cases. In our case series, gross or microscopic findings associated with dehydration or visceral gout were not described. Errors in diet formulation and errors in mixing feed ingredients at feed mills could be attributed as potential causes of salt toxicity in 5/7 cases in which high feed sodium levels were detected above the recommended level by the NRC for chickens (5). Additional findings of the analysis of ST diagnostic cases will be presented in a peer-reviewed publication.

ACKNOWLEDGEMENTS

We thank the staff of CAHFS Turlock branch and CAHFS Davis branch for providing technical assistance in the ancillary tests of this work.

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THE INTESTINAL MICROBIOTA OF "NORMAL" CHICKENS

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INTRODUCTION

After characterization of the microbiota by 16S rRNA gene sequencing started to be a mainstream method about 10 years ago, the intestinal microbiome has become a parameter that is frequently included in all kinds of *in vivo* chicken studies. It seems most relevant in trials testing feed ingredients, feed additives or intestinal pathogens, but has also been used in trials investigating pathogens causing lesions in other organs, heat stress or lighting programs. The reasoning is that all these factors, and more, influence the composition of the intestinal microbiota and that the intestinal microbiota in turn is crucial for the wellbeing and productivity of the birds. However, the myriad of factors that influence the microbiota and the myriad of bacteria that can colonize the intestinal tract make interpretation of the results difficult and limits it to comparison between groups. This approach does not allow conclusions if observed changes are good, bad, or ugly. The purpose of this study was to compile and analyze the intestinal microbiota of chickens that were left untreated, uninfected, and otherwise kept close to standard conditions to explore if it might be possible to define a "normal" intestinal microbiota.

MATERIALS AND METHODS

Available raw data of 16S rRNA gene Illumina sequencing of the intestinal microbiota of control groups of chicken trials were downloaded from the NCBI Sequence Read Archive. Inclusion criteria for data sets were, besides data availability and use of 16S rRNA gene sequencing on the Illumina platform that the birds from which the samples were taken, were untreated in a broad sense and raised in a controlled environment. Datasets of duodenal, jejunal, ileal, cecal and fecal samples were analyzed separately using a standard workflow including Qiime 2 and the phyloseq package in R. Investigated parameters were identified taxa including frequently encountered core taxa, alpha diversity, and beta diversity. The impact of geographic location, type, and age of the birds, housing, i.e. if birds were kept on wire or solid floor, and the sequenced region of the 16S rRNA gene were investigated. Differences in alpha diversity between these groups were tested for significance by Kruskal Wallis test. Differences in beta diversity were tested by multifactorial PERMANOVA.

RESULTS

A total of 86 data sets with 4472 individual samples were identified and included in the analysis. This included 142 duodenal samples from six data sets, 353 jejunal samples from 18 data sets, 1260 ileal samples from 28 data sets, 2148 cecal samples from 70 data sets, and 569 fecal samples from nine data sets. The V3-V4 region was most frequently used, followed by only the V4 region.

Preliminary analysis using selected cecal samples showed that 18 genera were present in more than 50% of samples in more than 50% of the experiments and might thus be regarded as core taxa. Sixteen of these were uncultured species in the class Clostridia, most frequently in the family *Oscillospiraceae* and *Lachnospiraceae*. The other two species were an unknown species in the genus *Escherichia* and interestingly *Lactobacillus salivarius*. There were significant differences in alpha diversity and beta diversity between experiments. Some alpha diversity measures were influenced by geographic location and bird type. PERMANOVA did not identify a significant influence of the included factors.

CONCLUSION

The results emphasize the high variability of the intestinal microbiota, which will make it more difficult to find indicators of a normal microbiota. On the other hand, the results also demonstrate that the investigated factors mostly do not systematically influence the investigated parameters, so if indicators of a normal microbiota can be identified, they will likely apply to a wide range of conditions. Future analyses include an analysis of the metabolic pathways present in the microbiota and comparison with treated groups.

(The full manuscript will be submitted for publication to *Poultry Science*.)

MODELING EARLY *ENTEROCOCCUS CECORUM* INFECTIONS IN POULTRY: EXPLORING HORIZONTAL TRANSMISSION EFFECTS ON COLONIZATION, EXTRAINTESTINAL TISSUE LESIONS, AND GROWTH PERFORMANCE

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SUMMARY

Enterococcus cecorum (EC) has recently been linked to pericarditis, septicemia, and early mortality in young broiler chicken flocks. Previously, our group demonstrated that *in ovo* administration with virulent EC at d18 of embryogenesis negatively impacted early performance and affected organs similar to current reports in the field. Since there is limited information regarding the prevalence, transmission, and pathogenesis of early EC infections, the current investigation focused on developing a horizontal transmission model to simulate exposure to virulent EC during the hatching phase. Results suggested that exposure to EC via direct injection into the amniotic cavity during late embryogenesis or the hatching phase as infected chicks emerge from the egg, may increase EC colonization, promote extraintestinal lesions, and impact performance depending on the strain and level of exposure.

INTRODUCTION

EC infections in broiler chickens have been traditionally associated with enterococcal spondylitis in older flocks (1, 2). More recently, morbidity and mortality associated with EC in young broiler chicken flocks has become an important issue (personal communications). Gross lesion development related to EC-associated sepsis, such as fibrinous pericarditis and focal heart necrosis, may also be observed in birds that do not appear to be clinically affected (3). There is limited research investigating the virulence of EC isolates obtained from these affected birds in the field. Recent studies conducted by our group highlighted the differences in pathogenicity and early performance impacts between these field strains (4). There is a need for a horizontal transmission model simulating exposure to EC during late embryogenesis to evaluate novel antibiotic alternatives to mitigate EC-associated morbidity and mortality in broiler chickens. The purpose of this study was to develop and validate a model replicating horizontal transmission of virulent EC strains during the hatching phase to simulate commercially relevant challenges associated with EC-associated systemic disease in a laboratory setting.

MATERIALS AND METHODS

Two experiments were conducted. In the preliminary study (Exp 1) to assess EC colonization at hatch, a subset of the embryos were inoculated by in ovo injection into the amniotic cavity (~10^4 CFU/200 µL/embryo) at d19 of embryogenesis with EC5, EC7, or EC11B (seeders or S) and comingled with non-infected embryos (contacts or C) in respective hatch cabinets. Treatment groups included 1) non-challenged control (NC), 2) EC5-C, 3) EC5-S, 4) EC7-C, 5) EC7-S, 6) EC11B-C, and 7) EC11B-S. At day-of-hatch, hatchability and livability was recorded and gastrointestinal tracts, liver, spleen, and vertebrate samples were collected to evaluate EC colonization at hatch. Samples were plated onto Chromagar Orientation as a selective media and incubated for 24 hours at 37°C in a CO2 enriched atmosphere to determine presumptive EC recovery (Log10 CFU and incidence) and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to confirm results. In Exp 2, chicks were placed in battery cages for the duration of the study. Pen weights were recorded at day-of-hatch, d7, d14, d21, and d26 to calculate average body weight gain (BWG). Samples mentioned above were collected at hatch, d7, d14, d21, and d26 post-hatch and macroscopic lesions were recorded at the time of necropsy for each group. Samples were also collected from affected organs for histopathology. ANOVA was used to determine significant differences at $P<0.05$ for BWG and EC recovery (Log10 CFU/g) with means being further separated using Student's t test. Chi-square was used to evaluate significant differences for

hatchability, macroscopic lesions, and EC incidence between NC and treatment groups.

RESULTS

In both experiments, hatchability was unaffected for all treatment groups. In Exp 1, EC colonization of the gut, liver/spleen, and vertebrate was elevated at hatch for all groups compared to NC indicating horizontal transmission was successful. In Exp 2, BWG was significantly ($P<0.05$) reduced for EC7-S across all time points evaluated compared to NC. BWG for EC5-S, EC5-C, and EC7-C from d0-21 and d0-26 was significantly lower than NC (Figure 1). However, there were no differences in BWG for EC11B-S and EC11B-C compared to NC throughout the study. No EC was recovered from the NC group at any time point evaluated. At hatch, EC recovery was significantly higher in the gut in EC7-C and EC11B-S compared to NC whereas only a numerical increase in other challenged groups was observed compared to the NC (Figure 2). At hatch, treatment groups EC5-S and EC7-S had a significantly higher incidence of EC recovery from the liver/spleen compared to NC with no significant differences in EC recovery or incidence from the vertebrate. Although EC was detected in free thoracic vertebrate (FTV) samples at all time points evaluated, there were no significant differences in EC recovery from the FTV until d26. At d26, EC5-S, EC7-C, EC7-S, and EC11B-C had markedly higher EC recovery (Log10 CFU/g) compared to the NC. Incidence of EC recovery from the FTV was significantly elevated for all challenged groups compared to NC at d26. At d7, d14, d21, and d26, incidence of EC recovered from the spleen was markedly higher in EC5-S compared to NC throughout the study whereas recovery from EC5-C was only significantly elevated at d21. Incidence of EC recovered from the spleen of EC7-S was statistically higher at d7, d14, and d26. However, a significant increase was observed at only d21 and d26 in EC7-C. At d14, there was a higher incidence of EC recovered from the liver in EC5-S compared to NC whereas only EC5-C had increased incidence in EC recovered at d21. Both EC7-C and EC11B-S had an elevated incidence of EC recovered from the liver compared to NC at d26. The liver, spleen, and heart were evaluated at necropsy to assess macroscopic lesion development. The prominent lesions observed throughout the study included focal heart necrosis, hydropericardium, splenomegaly, and hepatomegaly, with heart involvement being the most consistent organ affected across all treatment groups compared to NC.

CONCLUSION

These results indicate that exposure to EC via direct injection into the amniotic cavity during late embryogenesis or during the hatching phase as infected chicks emerge from the egg, could hinder early performance in broiler chickens, and increase EC colonization and translocation to the gut, liver, spleen, and FTV. Additionally, exposure to EC during the hatching phase may result in tissue/organ damage, and ultimately impact early performance in broiler chickens. This horizontal transmission model could be used to evaluate strategies to mitigate EC infections pre- and post-hatch.

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

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Figure 1. Average BWG from d0-26 (Exp 2). Data expressed as mean ± standard error. ANOVA used to determine significant differences between treatment groups with means being further separated using Student's t test. [a-c] Indicates significant differences at ($P < 0.05$) between the treatments.

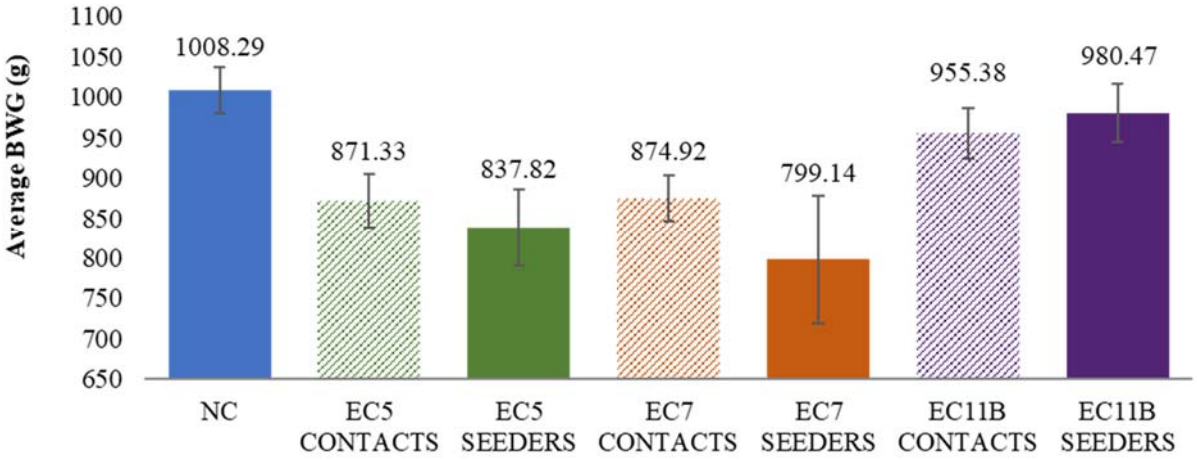
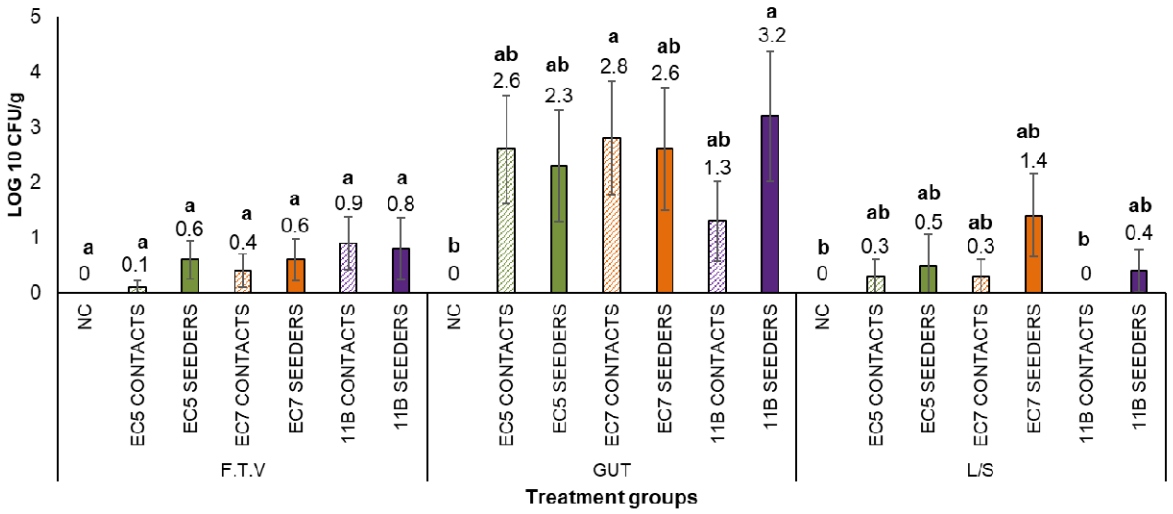


Figure 2. EC recovery (Log10 CFU/g) from the FTV, gut, and liver/spleen (L/S) samples collected at hatch (Exp 2). Data expressed as mean ± standard error. ANOVA used to determine significant differences between treatment groups with means being further separated using Student's t test. [a-c] Indicates significant differences at ($P < 0.05$) between the treatments.



COMPARISON OF THE CROSS PROTECTIVE ABILITY OF MA5 AND DMV/1639 TO BRONMASS AND IBRON INFECTIOUS BRONCHITIS VIRUS VACCINES AGAINST MULTIPLE CHALLENGE VIRUSES

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SUMMARY

Vaccination strategy for control of infectious bronchitis virus (IBV) has evolved from monovalent homologous protection to multivalent cross protection. The purpose of this study was to evaluate the cross-protective ability of Ma5 and DMV/1639 vaccines in comparison to iBron and BronMass vaccines against four different challenge viruses: CA/1737, GA08, DMV/1639, and GA13. Vaccine takes were poor for BronMass, but very good for all others. There was no difference in clinical sign scores between any of the experimental groups, and all had lower scores than the non-vaccinated controls. All vaccinated groups had significantly lower viral loads than the non-vaccinated and challenged controls, and for 3/4 challenge viruses there was no difference in viral loads between vaccinated groups. The only difference seen was the DMV/1639 challenge group, where the group vaccinated with Ma5 and DMV/1639 had a significantly lower viral load post challenge than the iBron and BronMass vaccinated group.

MATERIALS AND METHODS

Viruses. The MA5 Mass type (Merck Animal Health), iBron GA08 type (Ceva), BronMass Mass type (Ceva) and in development (our laboratory) DMV/1639 type IBV vaccines will be used in this study. The challenge viruses used are GA08/GA08/08, GA/13384/2013, DMV/1639/11, and CA/1737/04.

Experimental design. Three different groups of 100 broiler chicks each will be used in this study. In Group 1, chicks will be spray vaccinated with Ma5 and DMV/1639 vaccines at full manufacturer's dose. In Group 2, chicks will be sprayed with iBron and BronMass at full manufacturer's dose. Chicks in Group 3 will remain unvaccinated and serve as controls. 100 chicks are not needed for control, but to keep stocking density the same between groups they will all be placed. Each group of chicks will be placed and reared in individual colony houses on fresh litter to mimic commercial housing conditions. Chicks in vaccinated groups will be swabbed in the choanal cleft on day 7 post-vaccination to evaluate vaccine infection and replication, and then again on day 28 prior to challenge to evaluate vaccine persistence. On day 28 post-vaccination, 45 birds from each vaccinated group will be moved to isolator units and 10 birds each will be challenged with one of four challenge viruses. Five additional birds will be kept unchallenged as controls. Additionally, 25 birds from the non-vaccinated group will be moved to isolator units and 5 birds each will be challenged with one of four challenge viruses. Five additional birds from this group will be kept unchallenged as controls. Five days post challenge, birds will be evaluated for clinical signs and swabbed in the choanal cleft for PCR evaluation of viral load post-challenge.

RNA extraction and challenge virus detection by real time RT-PCR. After swabbing, swabs will be placed into 1 mL of ice-cold PBS and stored at -80C until processed. Viral RNA will be extracted from 50ul of swab fluid using the MagMAX -96 RNA Isolation Kit (Ambion Inc., Austin TX) according to the manufacturer's protocol on a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA). Real time RT-PCR will be conducted using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA) and the AgPath-ID™ One-Step RT-PCR kit (Ambion Inc.) according to the manufacturer's recommendations. Primers and probe for the real time RT-PCR will correspond to the specific serotype being tested for. The primers will be obtained from Integrated DNA Technologies (Coralville, IA) and Taqman^{Ò}probe was synthesized by BioSearch Technologies (Novato, CA).

Clinical Signs. Clinical signs will be recorded and scored based on our published laboratory scoring method where a 0= no signs, 1= slight wheezing or snicking, 2= more pronounced wheezing, sinus exudate, conjunctivitis, and 3= rales.

RESULTS

Three hundred chicks were selected from the hatch and divided into three groups of 100. The first set of 100 chicks was immediately placed into a colony house and constituted the non-vaccinated group. The second set of 100 chicks was vaccinated with iBron and BronMass vaccines from Ceva using a Merck SprayCox II commercial hatchery spray cabinet. Chicks were allowed to dry (~15 minutes) and were then placed into a colony house for rearing. The vaccination room and cabinet were cleaned and sanitized, and then the last group of 100 chicks was spray vaccinated in the same manner using the Ma5 and DMV/1639 from Merck Animal Health. The DMV/1639 vaccine used was a vial of the special permit live-attenuated vaccine currently being produced by Lasher Laboratories. All chicks were reared on fresh litter and had ad libitum access to feed and water.

Seven days post-vaccination, the vaccinated groups were swabbed in the choanal cleft to evaluate vaccine takes (Figure 1). As expected, both viral loads and percent positive birds were excellent for both Ma5 and DMV/1639 vaccines. Average viral load for both was ~26 Ct value, and over 90% of birds were positive for each. For the iBron and BronMass group, the viral load and percent positive birds were excellent for iBron but not the BronMass vaccine. iBron had an average viral load of ~27Ct value and greater than 95% of birds positive for vaccine virus. The BronMass group had much fewer birds positive (~35%), and the positive birds had a lower average viral load (~31 Ct value) than the other vaccines.

Twenty eight (28) days post-vaccination birds from each vaccinated and non-vaccinated group were moved to isolators for challenge per the experimental design in Table 1. Birds were challenged with one of four challenge viruses or not challenged as a control. Five days post challenge, protection was evaluated by clinical signs and viral load from choanal cleft swabs. For the CA/1737/04 and GA13 challenged groups, the clinical sign scores in the non-vaccinated group were not severe, so there was no significant difference between clinical sign scores in any group. For the GA08 and DMV/1639 challenged groups, clinical sign scores in all groups (vaccinated and non-challenged and vaccinated and challenged) were significantly lower than the non-vaccinated and challenged control. There was no difference in clinical sign score between the vaccinated groups challenged with GA08, and only a slight numerical difference in clinical sign score between the vaccinated groups challenged with DMV/1639.

Viral load data is shown in Figure 2. For both vaccinated and non-challenged groups, some vaccine virus could still be detected at 33 days of age (5 days post challenge). Not all samples were positive for vaccine virus, and average viral loads were very low (~34-36 Ct value). This is expected with both the iBron and DMV/1639 vaccines and very representative of what is seen in broiler surveillance in the commercial industry. For all challenge virus types, the vaccinated and challenged groups had significantly lower viral loads post challenge than the non-vaccinated and challenged control group. There was no significant difference between the vaccinated and challenged groups regardless of vaccine combination or challenge virus, with one exception. For the DMV challenge, the Ma5 and DMV/1639 vaccinated group had a significantly lower viral load post-challenge than the iBron and BronMass vaccinated group. There were slight numerical differences in average viral load between the vaccinated groups for other challenge viruses, but nothing significant.

DISCUSSION

The purpose of this study was to evaluate the cross-protective ability of the Ma5 and DMV/1639 vaccine combination in comparison the iBron and BronMass vaccine combination against different challenge viruses. A general review of the data shows that there wasn't a dramatic difference between the vaccine combinations, but there are some distinct small differences. First and foremost, the vaccine take data was markedly different between the Mass type vaccines. Both the DMV/1639 and iBron vaccines are known for being highly effective at infection and replication after spray vaccination, and the data from this trial was no different. The same can be said for the Ma5 vaccine. Alternatively, the BronMass vaccine has not shown the same level of vaccine takes and this was evident in this study. It would stand to reason that utilizing this vaccine in a multi-valent, cross protection vaccine program would not be recommended as the immune response to this vaccine would be poor because of subpar infection and replication. This is not exactly what was seen in the challenge data, however. There was no difference in clinical sign scores between any of the experimental groups, and all groups had lower clinical sign scores than the non-vaccinated and challenged control groups. This highlights a concept that we have discussed and several publications have proven that almost any vaccine or vaccine combination (with a few caveats) can reduce clinical signs in experimental settings. This also highlights that clinical sign scores are a not an extremely sensitive metric for evaluating protection and should always be compared in addition to other factors, especially viral loads after challenge. When evaluating viral loads post-challenge, again, all

vaccinated groups had significantly lower viral loads than the non-vaccinated and challenged control group and for 3/4 challenge viruses, there was no difference in viral loads between any of the vaccine combinations used. The only difference seen was for the DMV/1639 challenge group where the group vaccinated with Ma5 and DMV/1639 had a significantly lower viral load post challenge than the iBron and BronMass vaccinated group. This is expected since the DMV vaccine is homologous to the DMV challenge virus however, the same was not seen for the iBron (GA08 type) vaccinated and GA08 challenged group. This could be partially due to the lack of the broader immune response from the poor infection and replication of the BronMass vaccine, but also lends validity to the cross-protective ability of the Ma5 and DMV/1639 vaccine combination.

Figure 1. Post vaccination qRT-PCR data.

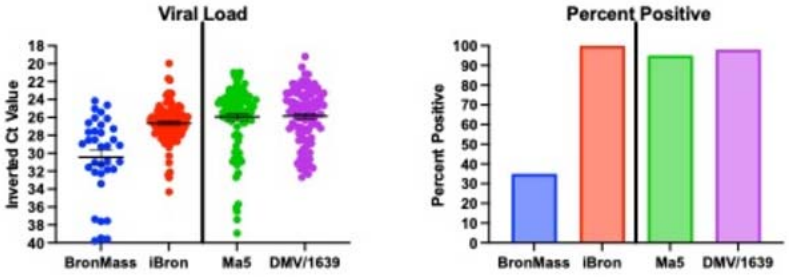
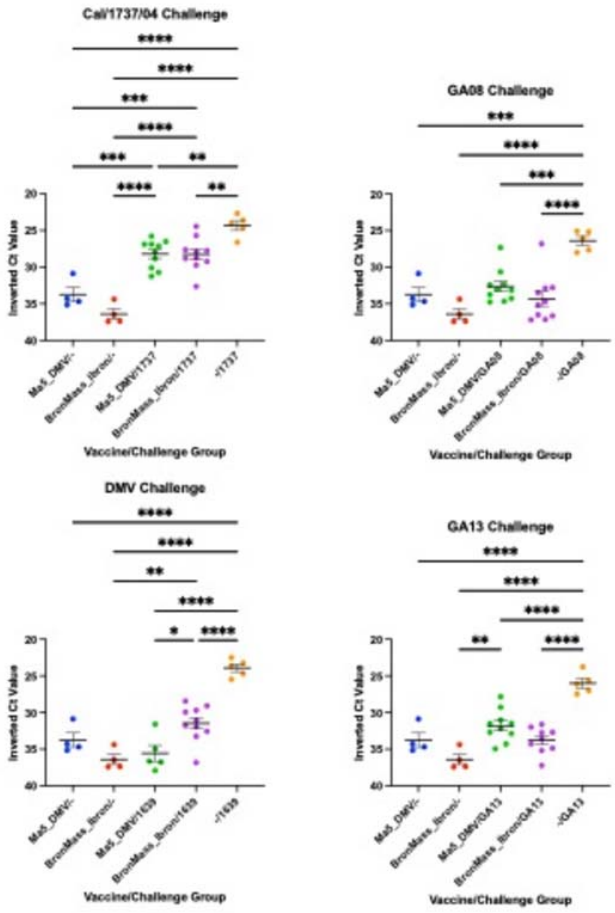


Figure 2. Viral loads post challenge represented as Ct values by qRT-PCR.



ANALYSIS OF GEL-PAC® AS A DILUENT FOR COMBINED INFECTIOUS BRONCHITIS VIRUS AND COCCIDIA VACCINE APPLICATION VIA GEL-DROP

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SUMMARY

This study evaluated the stability of vaccines against infectious bronchitis virus (IBV) and coccidiosis in commercial poultry when combined and administered via aerosolized water spray or gel-drop diluents, both *in vitro* and *in vivo*. Diluents were compared for their impact on IBV vaccine thermal stability, IBV vaccine titer stability, coccidiosis vaccine positional stability throughout the application process, hatchling chick body temperature, and coccidia vaccine cycling pattern uniformity. Diluents did not differ in effect on chick thermal response or IBV vaccine stability. Gel-drop diluent provided more stable coccidia oocyst suspension without agitation during vaccination, and improved vaccine oocyst uniformity during post-vaccination cycling. Gel-drop proved at least as effective as traditional water spray for delivering the IBV and coccidia vaccines used in this study, both alone and together in a single vaccine suspension.

INTRODUCTION

Infectious bronchitis virus (IBV) and coccidia vaccines have been traditionally administered by aerosol spray cabinet in the hatchery at one day of age. IBV is an upper respiratory tract pathogen, therefore exposing the IBV vaccine to upper respiratory tissue (conjunctiva, Harderian gland, choanal cleft, trachea) is critical for proper vaccination and immunization. Application volumes of IBV vaccines vary, but typically range from 7-21 mL per 100 chicks (1 chick box). Unlike IBV, coccidia is an enteric pathogen, making vaccine ingestion critical to deliver the oocysts to the target tissue in the gut. Recently introduced gel-drop vaccination technology has not been thoroughly tested for all vaccine types (respiratory vs. enteric). Gel-drop vaccine diluent is intended to increase coccidia vaccine application efficiency via direct ingestion. The gel-drop mechanisms utilize a highly viscous gel applied under pressure through an application bar with openings of varying size which release the gel. The gel then "streams" out of the openings and forms droplets before reaching the chicks. Ideally the chicks then preen the droplets, ingesting the coccidia oocysts at the same time. With the recent introduction of new coccidia vaccine formulations, designed to permit combining with other vaccines during application (IBV most notably), the question arises whether IBV vaccines can be effectively applied via gel-drop, combined with coccidia vaccine, in the same manner.

MATERIALS AND METHODS

In vitro *Assessment of Gel Effect on Vaccines*

IBV vaccine was mixed with Gel-Pac® (GP) in the recommended concentration using room temperature water (21°C) by reconstituting powdered gel, then adding vaccine. A sample of the gel/IBV vaccine mixture was then taken immediately after mixing and every thirty minutes for four hours. These samples were titered in embryonated eggs to evaluate the stability of IBV vaccine in the gel over time. A water/IBV vaccine mixture was also titered in the same way at the same concentration as a control. In a second experiment, chilled water (13°C) was used to reconstitute the GP and the experiment repeated, using a chilled water/IBV vaccine group as a control. It has been previously shown that IBV vaccine needs to be kept cool (<18°C) to maintain titer over time, and this experiment tested any differences from that for the gel. After the temperature effects for the water to be mixed with the gel were evaluated, the next experiment involved mixing IBV and coccidia vaccines together in a single gel-drop vaccine and repeating the experiment. Previous studies have shown that there is no effect of IBV vaccine on coccidia oocysts, so the only evaluation was to measure the IBV vaccine titer over time. Lastly, the coccidia vaccine was only mixed in the gel and held with no further mixing to establish the positional stability of oocysts in suspension for each diluent. Samples were taken from the top 1/3, the middle 1/3, and the bottom 1/3 layer of the vessel immediately after mixing and then

every 30 minutes for at least two hours. These samples were then floated and coccidia vaccine oocysts were counted. A coccidia vaccine mixed in water group was prepared in the same way and sampled as a control.

In vivo *Assessment of Gel Effect on Vaccines*

Diluent effect on chick body temperature. Newly-hatched chicks were placed into chick baskets prior to vaccination and allowed to acclimate to the environment of the vaccination room. Temperatures of 25 chicks were measured by rectal thermometer before vaccination to obtain baseline data. After vaccination, the temperature of 25 chicks from each vaccinated group were measured throughout 60 minutes to evaluate any reductions and/or recoveries in body temperature from the vaccination process.

Diluent effect on IBV vaccine titer. Four groups were vaccinated using commercial hatchery aerosol spray or gel application vaccination equipment.

Group 1 - One hundred broiler chicks were spray vaccinated using dual fan nozzles at a full dose with IBV vaccine alone, mixed in water. All 100 chicks were held in the chick box for 1 hour and then placed in a colony house following vaccination.

Group 2 - One hundred broiler chicks were spray vaccinated using dual fan nozzles at a full dose with IBV and coccidia vaccine combined in water. All 100 chicks were held in the chick box for 1 hour and then placed in a colony house following vaccination.

Group 3 - One hundred broiler chicks were vaccinated using gel at full dose with IBV vaccine alone. Vaccine and gel were mixed according to the manufacturer's instruction. All 100 chicks were held in the chick box for 1-hour post vaccination to allow for preening and monitoring and then placed in a colony house.

Group 4 - One hundred broiler chicks were vaccinated using gel at full dose with IBV and coccidia vaccine combined. Vaccine and gel were mixed according to the manufacturer's instruction. All 100 chicks were held in the chick box for 1-hour post vaccination to allow for preening and monitoring and then placed in a colony house.

IBV vaccine infection rate and viral load were evaluated by swabbing every chick remaining in a colony house from each group on days 5 and 7 post-vaccination. All chicks were euthanized after their respective sampling period.

Diluent effect on coccidiosis vaccine shedding. For all groups receiving coccidia vaccine, twenty chicks from each group were removed from the colony houses on day 4 and placed individually into isolators. They were held there for 5 days (days 5-10 post-vaccination). Feces from each chick were collected daily and oocysts were counted to evaluate infection rate and oocyst numbers shed per gram of feces.

Vaccines. Commercially available IBV (Ma5) and coccidia vaccine (B-52) from Merck Animal Health were used.

IBV vaccine detection post-vaccination. At five and seven days post vaccination, all chicks were swabbed in the intrachoanal cleft, and qRT-PCR were performed on all samples.

Virus detection. Samples were tested for IBV by quantitative real time RT-PCR and expressed as the relative amount of virus (cycle threshold (Ct) value) in the sample, as well as viral genome copies. Viral RNA was extracted from each sample using the MagMAX-96 RNA Isolation Kit (Ambion Inc., Austin TX) according to the manufacturer's protocol on a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA) and used as template in the reaction. Real time RT-PCR was conducted using an Applied Biosystems Fast 7500 Real Time PCR Machine (Life Technologies, Carlsbad, CA) and the AgPath-ID™ One-Step RT-PCR kit (Ambion Inc.) according to the manufacturer's recommendations. Primers and probe for the real time RT-PCR were previously published and consist of a forward primer IBV5'GU391 (5'-GCT TTT GAG CCT AGC GTT-3'), a reverse primer IBV5'GL533 (5'-GCC ATG TTG TCA CTG TCT ATT G-3') and a Taqman® dual-labeled probe IBV5'G probe (5' –FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3'). The primers were obtained from Integrated DNA Technologies (Coralville, IA) and the Taqman probe was synthesized by BioSearch Technologies (Novato, CA). Real time RT-PCR components and thermocycler parameters were conducted as previously described.

Oocyst enumeration. Oocysts were enumerated for all parts of the trial utilizing a McMaster's chamber. Feces was collected and saturated in 10-times water and allowed to soak overnight to release oocysts. Fecal slurry was filtered through a double layer of cheese cloth, and the flow through was centrifuged to concentrate oocysts. Sample was then mixed with an appropriate dilution of saturated salt water. The resulting sample was then mixed and pipetted into a McMaster's chamber. The chamber was held for three minutes so oocysts could rise to the top of the chamber, then oocysts were counted using the method of Conway and McKenzie. Oocysts were speciated according to the morphological characteristics of the different species present in the vaccine according to the manufacturer, including size and shape.

RESULTS

In vitro *Assessment of Gel Effect on IBV Vaccine*

IBV vaccine was mixed with GP gel made with room temperature (21°C) water or chilled (13°C) water and titrations were performed on the mixture initially, then every 30 minutes for 4 hours to assess longevity of IBV vaccine over time in gel. In addition, IBV vaccine and coccidia vaccine were mixed with GP at the same two temperatures and IBV titrations were again performed.

Overall, minor variations in individual titers at various timepoints proved transient, resulting in no difference in IBV vaccine titer over the 4-hour period when mixed alone in GP, whether at room temperature or chilled. To account for variability that may have been induced by using separate vaccine vials for each treatment, titer changes from each treatment's initial input were calculated. When evaluating titers of IBV vaccine mixed with coccidia vaccine, there was a pattern of slight decline in IBV titer from time 0 to 1 hour (less than 1 log), then a stabilization of IBV titer from the 1-hour to the 4-hour timepoint in all groups. The decline from 0 to 1 hour in all IBV plus coccidia groups was not seen in the IBV only groups and therefore may be attributed to mixing with coccidia vaccine (Figure 1). The decline in IBV titer in the presence of coccidiosis vaccine was apparently unrelated to diluent temperature or gel effects.

Positional Stability of Coccidiosis Vaccine in Water and Gel Suspension

When mixed with water and not continually stirred, the oocysts from the coccidia vaccine settled to the bottom of the vessel almost immediately and stayed there. The oocyst distribution in the vessel was uniform at time 0 immediately after mixing, but by the end of 30 minutes without agitation the top and middle fractions were devoid of oocysts. Concurrently, oocysts concentrated in the bottom fraction of the mixture increased. This matches previous data from this lab and concurs with the manufacturer's recommendations to constantly mix coccidia vaccine in water diluent.

Conversely, coccidia vaccine mixed with GP experienced very little settling over time. There was a passing decline in oocyst numbers in the top third of the vaccine solution between 0 and 30 minutes, and a correlated increase in the middle fraction at this time point, followed by increasingly uniform oocyst distribution throughout 120 minutes in all fractions of the mixture. Despite the slight initial shift in distribution, over the 2-hour duration the concentration of oocysts remained more uniform in GP than water.

In vivo *Diluent Effect on IBV Vaccine Titer*

Evaluating the *in* vivo *effect of* GP on the vaccines used four different groups of chicks: Group 1 - IBV vaccine alone via water spray; Group 2 - IBV and coccidia vaccines mixed together via water spray; Group 3 - IBV vaccine alone via gel drop; and Group 4 - IBV and coccidia vaccine mixed together via gel drop. IBV infection and replication rates were determined from post vaccination swabs at 5 and 7 days. Coccidia vaccine oocyst shedding number and patterns were evaluated in 20 chicks from Groups 2 and 4 that were placed in isolators for fecal collections.

All IBV vaccination methods proved successful. At 5 days post-vaccination, the mean qRT-PCR Ct values for all treatments, a measure of viral load, was 29 and below. Additionally, there was no difference in the percent of chicks positive for IBV between any groups (IBV alone in water 94%; IBV plus coccidia in water 95%, IBV alone in gel 93%, IBV plus coccidia in gel 95%) indicating successful vaccination across all treatment methods. Ultimately at 7 days post vaccination, the difference in mean Ct values improved further, reducing the average Ct range to 25-26, with no difference between groups in percent of chicks infected at this timepoint. Furthermore, day 7 Ct variability was less than on day 5, indicating IBV vaccine uniformity via replication continued to improve.

In vivo *Diluent Effect on Coccidiosis Vaccine Oocyst Number and Shedding Pattern*

The overall oocyst pattern for coccidia vaccine shedding in feces after vaccination via water spray and gel were similar, with peaks of shedding at 7 days post vaccination (Figure 2). The major difference between the two groups was the uniformity of the oocyst counts. Oocysts per gram of feces in the GP group increased steadily from day 5 to 7 (SD ranging 501 to 11,356), whereas those in the water spray group spiked on day 7 post vaccination (SD ranging 5100 to 33,464). Examination of the raw data revealed two birds vaccinated by water spray were shedding many more oocysts at 77,000 and 91,000 per gram on day 7, which skewed the data dramatically and introduced much larger variation at that time point compared to other days and the GP group. For both applications, 90% of birds shed oocysts during the 5 days evaluated.

Chick thermal regulation

Overall, the chicks were warm when coming out of the hatchers and had slightly elevated chick temperatures. There was a decline in chick temperatures for both water and gel application groups, and the pattern of decline and subsequent stabilization was very similar, with no differences between the groups.

DISCUSSION

Spray vaccination of chicks in the hatchery has long been a standard production practice for the poultry industry. For respiratory vaccines like IBV, this route of administration, which mists aerosolized vaccine mixed in water onto chicks, seemed appropriate because the vaccine targets respiratory tissues such as the eyes and nares. For enteric vaccines like coccidia, which must be ingested to be protective, spray vaccination was not a natural fit but was adapted to work. As technology evolved in the poultry industry, so have vaccination methods and with that has come the introduction of edible gels for application of poultry vaccines. Unlike spray vaccination, gel vaccination utilizes droplets of gel containing vaccine "dropped" down onto chicks. Naturally, this edible gel containing vaccine seems like a very appropriate way to apply enteric biologicals, including coccidia. Hatcheries sometimes desire to combine coccidia vaccine with respiratory vaccines in a single gel application, but the practice does not intuitively result in respiratory vaccine contact with eyes and nares of a chick. Instead, gel-delivered respiratory vaccine protection relies on oropharyngeal contact to get into respiratory tissues, not unlike vaccinating via drinking water. The advent of gel application compels researchers to evaluate gel products' delivery of vaccines in comparison to previously used methods.

In this study, GP was evaluated for stable delivery of IBV and coccidia vaccines to commercial poultry, both *in vitro* and *in vivo.*The *in vitro* study tested the stability of IBV vaccine mixed in GP over time, with temperature being the confounding variable. The present study used a commercial IBV vaccine mixed in GP gel made with room temperature water and compared that to IBV vaccine mixed in room temperature water only. The experiment was repeated with GP gel made with chilled water compared to IBV vaccine in chilled water alone.

Overall, there was no difference in titer at the initial timepoint or in the trend over the 4-hour test period between any group tested regardless of diluent (GP gel vs water) or temperature. Any differences in titer at individual timepoints were within ranges previously encountered when working with a gel diluted IBV vaccine. The data indicates that GP gel does not negatively impact IBV vaccine livability or infection rate *in vitro.*

Gel diluents provide a seemingly more natural route of administration for enteric pathogens or vaccines that need to be ingested (such as coccidia) than a water solution that is aerosolized. Additionally, water-based dilutions of coccidia vaccines must be continually mixed to prevent settling of the heavier, denser coccidia oocysts in the vaccine over time. It is expected that a gel diluted coccidia vaccine solution, being more viscous than water, would have the capability to keep oocysts in suspension over time. Testing the capability of GP to maintain a uniform coccidia suspension, coccidia vaccine was mixed with water or GP gel and held undisturbed for 2 hours. Samples collected from the top 1/3, middle 1/3, and bottom 1/3 of each solution every 30 minutes highlighted that nearly all oocysts in the water solution migrated from the top and middle portions of the vaccine solution and settled to the bottom of the flask within 30 minutes. A large portion of the oocysts that settled to the bottom of the flask were not retrievable via pipette. Any apparent increase in oocyst numbers in the bottom and middle fractions over time corresponds to oocysts being jarred from the bottom during handling for sample collection and equilibrating to the diluent concentration. Conversely, oocyst counts in the GP solution, while experiencing variation at the 30-minute timepoint, were ultimately well distributed, with oocyst counts being more uniform throughout the top, middle, and bottom of the gel suspension for 2 hours. This leads to the conclusion that, provided the coccidia vaccine is evenly mixed into the GP gel initially, the gel suspends the oocysts without settling over the course of normal vaccination times.

Noting the *in vitro* studies in this series showing that IBV vaccine is stable in GP gel, and the GP gel keeps coccidia oocysts in suspension over time without continued mixing, another investigation mixed the IBV and coccidia vaccines together in GP gel to examine the possibility of any interactive effect. Like the IBV only experiment, IBV and coccidia vaccines were mixed into water or GP gel solutions made with different temperature water and samples were taken for IBV titrations over 4 hours. All groups behaved the same in terms of titer stability over time. A slight IBV vaccine titer decline over time is consistent with other reports of mixing IBV and coccidia vaccines, and this is not unexpected. This data shows that combining the specific IBV and coccidia vaccines used in this study in GP gel is feasible; the IBV vaccine stability was not compromised when combined with coccidia vaccine in GP, remaining in a protective range.

The final experiments in the series tested the GP gel diluent in chickens to collect live animal data. Four groups of chicks were vaccinated with either IBV vaccine alone in a water diluent by spray, IBV vaccine alone in the GP gel via gel-drop, IBV plus coccidia vaccines together in water diluent by spray, or IBV plus coccidia vaccines together in GP gel via gel-drop. Swabs were collected from all chicks in every group at 5- and 7-days post-vaccination to assess IBV

vaccine infection and replication. A subset of chicks from the coccidia vaccinated groups were housed individually from days 5-10 post-vaccination for fecal collection and oocyst enumeration.

Assessing IBV viral load by real-time PCR in chicks after vaccination, overall vaccine uptake and replication in chicks was excellent for all application methods and vaccine combinations, with all groups well above 90% chicks positive. All vaccination methods produced abundant vaccine virus replication. Relying on the 7-day post-vaccination PCR IBV vaccine detection, the standard practice in this laboratory, revealed all groups were nearly identical with an amply protective viral load regardless of application method or vaccine combination.

When evaluated for coccidia vaccine infection, replication, and shedding, a very characteristic shedding pattern was found in both coccidia vaccinated groups with a peak at 7 days post-vaccination. The OPG being shed in feces from the water spray group was much more variable on day 7, influenced by high oocyst counts from two individuals. In the poultry industry, consistency in shedding is critical. Coccidia vaccines used in the US are not attenuated and immunity is achieved through controlled dosing and repeat exposure. Vaccines are administered in low-doses, and these doses are amplified through replication in the bird. When administered properly, the amount shed in the first 7-8 days (first cycle of coccidia replication) is ideally low and evenly distributed among birds. This facilitates chicks re-ingesting the oocysts from the litter and obtaining a second small (albeit bigger than what was applied in the hatchery), uniform dose for a second round of infection and replication. When vaccine is applied in a less efficient manner, the chicks may be dosed non-uniformly which will lead to variable shedding after the first replication cycle of oocysts. Chicks that then re-ingest a very small number of oocysts from the litter will have a smaller second round of infection, but chicks that ingest an extremely large number of oocysts from the first shedding cycle will subsequently have an extremely large second round of infection and replication. When vaccines are not attenuated, the ingestion of a large bolus of oocysts (known in the industry as a cocci bomb) can lead to clinical disease caused by the vaccine. The two chicks in the water spray application group shedding over 70,000 oocysts per gram each represent this potential scenario.

Lastly, when evaluating the body temperature reduction experienced by chicks after vaccination, no difference was observed between the spray or gel applied groups. When chicks hatch, their internal temperature is higher than their equilibrium temperature as they have been in a hatcher at an elevated temperature for 12-36 hours. The longer they are out of the hatcher in lower environmental temperatures there is a natural decline in body temperature to equilibrium state (approximately 40°C). Chicks are vaccinated very soon after being pulled from hatchers, and the process of applying liquid to the chicks to deliver the vaccines accelerates this process to some degree. Often body temperatures following vaccination fall below normal equilibrium state but rebound to normal in most cases. This pattern was observed in this experiment as well. The drop in temperature is not the major issue with thermal stress, the more concerning issue is how long it takes for chick temperatures to return to equilibrium. This is much more influenced by the environment than anything else, with temperature, humidity, air movement, etc. being prime factors. These environmental parameters directly affect how quickly chicks can dry, thus impacting evaporative cooling. In this study, the chicks were held in the same environment for the duration of temperature collection with no direct air blowing on them. In this controlled environment, the chicks gel began to return to equilibrium temperature after 25 minutes. By this time, chicks began to settle down and compact within the box. This behavior facilitates body temperature increase. In a well-managed hatchery where temperatures and humidity are controlled for chick welfare, this would not be an issue. In areas or hatcheries where environmental control is difficult or external weather is extreme (hot or cold), excessively wetting chicks could pose an issue to chick health.

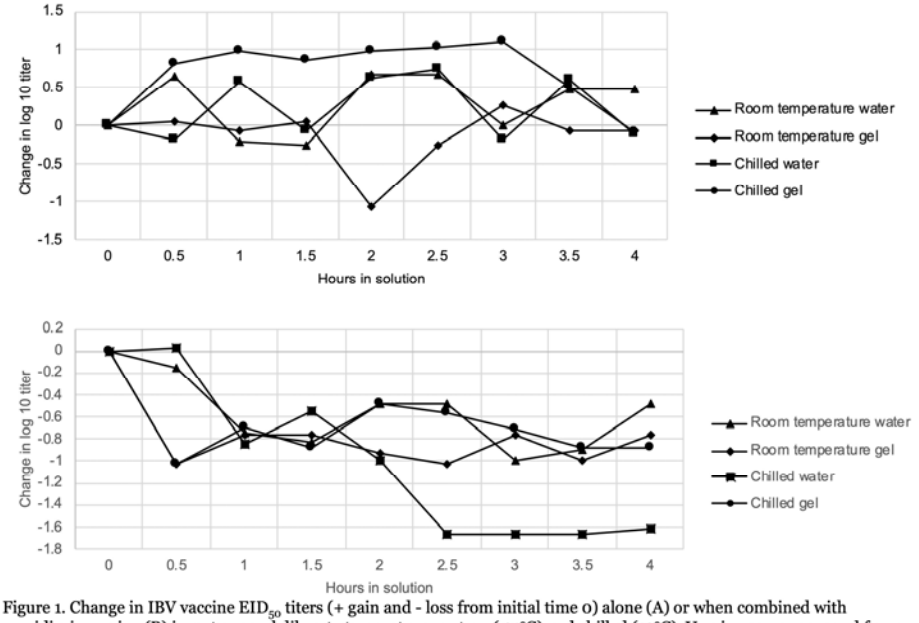

Figure 1. Change in IBV vaccine EID_{50} titers (+ gain and - loss from initial time 0) alone (A) or when combined with coccidiosis vaccine (B) in water or gel diluent at room temperature (21°C) and chilled (13°C). Vaccines were prepared from separate vials at time 0 and held for 4 hours, with samples extracted for titration each 30 minutes.

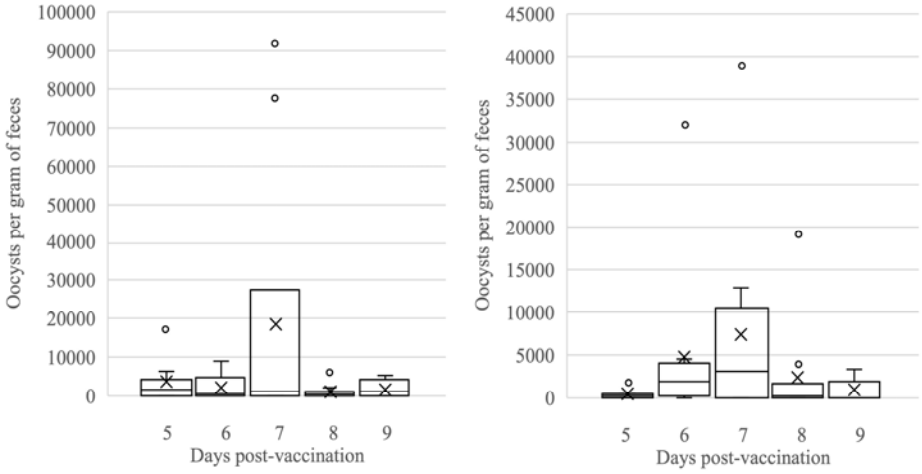

FIGURE 2. (A) Water spray and (B) gel drop coccidia vaccination shedding number and pattern in chick fecal samples. Symbol X represents mean of data, upper and lower limits of box envelop 50% of data, whiskers envelop the range of included data, symbol o represents individual outliers.

EARLY PROTECTION AGAINST INFECTIOUS BRONCHITIS VIRUS BY VARIABLE DOSES OF SUPPLEMENTAL PASSIVE ANTIBODIES

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SUMMARY

While infectious bronchitis virus (IBV) is primarily responsible for acute respiratory disease in chickens, urogenital disorders like false layer syndrome have also been associated with early IBV infection. We have previously proved the long-term protective effects of maternal protection. We have also seen that maternal antibodies present in commercially sourced chicks have a high variability in titers, which can allow breakthrough infections. Here, we investigate the protection provided by low and high dose spray application of hyperimmune serum to day-of-age chicks. Additionally, we evaluated both ocular-nasal and intramuscular challenge routes to examine if the protection seen by spray application of IBV antibodies is limited to the respiratory tract. These investigations are integral to showing the effectiveness and mechanisms of protection provided by supplemental application of antibodies.

INTRODUCTION

Infectious bronchitis virus, a gammacoronavirus, is responsible for acute upper respiratory disease in chickens. In addition to its respiratory presentation, IBV can also cause chronic nephrotic and reproductive issues, such as false layer syndrome (FLS), in long-lived birds. FLS is a disorder in which hens ovulate normally but do not lay. It has been long known that the incidence of FLS at maturity is negatively correlated with the age of exposure to IBV (1). It has also been shown that IBV maternal antibodies protect against cystic and oviductal atrophy, peritonitis, and egg production drops (2) as well as acute respiratory symptoms (3, 4). We have also recently found that the efficacy of maternal protection is dependent on the intensity of IBV challenge, that maternal antibody titers may not be uniform in commercial flocks at hatch, and reiterated that maternal antibodies disappear before 2 weeks of age (2, 4-6). Because the chicken immune system does not fully develop until around 30 days of age (7), it is important to investigate supplementation to maternal protection which can fill the window of time where chicks are immunologically underdeveloped. For these reasons, we investigated if hyperimmune serum can be applied for protection against IBV challenge, what application methods are most effective, the effect of dose, and the mechanism of protection provided by supplementation with IBV antibodies.

MATERIALS AND METHODS

Birds. For raising hyperimmune serum, twenty-two 52-week-old hens were obtained from the UCD MHC-B haplotype congenic chicken lines, 253 and 331, and raised on the floor. For the two trials, 506 fertile SPF eggs were hatched at the UCD Meyer Hatchery and raised in brooders and isolator units.

Virus and vaccines. Only Mass type IBV strains were used for all experiments. To raise hyperimmune serum, live M41 ($10^{3.5}$ EID_{50}/dose), formalin-inactivated M41 ($10^{7.5}$ EID_{50}/dose), and the Poulvac (Zoetis) IB Mass live vaccine (1X dose) were given in 10-day intervals beginning with ocular nasal application of live M41, 4 rounds of inactivated intramuscular application, one ocular application of Poulvac, and one additional inactivated IM injection. Challenge for both trials consisted of ocular nasal application of M41 strain ($10^{4.5}$ EID_{50}/dose).

Application method trial. A pilot study was performed to determine the appropriate application method of hyperimmune serum and if protection was possible. At 19-days of embryonation, 60 eggs were inoculated with 200 µL each of hyperimmune serum (~10,000 GMT). After hatch, 50 day of hatch chicks were sprayed with 10 mL of the same hyperimmune serum. Forty chicks were left untreated. At 7 DOA, half of each group received M41 challenge. At 5 and 10 DPC, respiratory signs and viral loads in choanal clefts and cloacas were assessed. At 21 DPC, necropsy was performed, sinus and airsacculitis scores assessed, tracheas and kidneys collected for histopathology, and tracheal washes, cloacal swabs, lungs, and kidneys were collected for viral load assessment.

Dosing and mechanism of action trial. After assessing the results of the above trial, low and high

dose applications of spray immunization and the route of challenge were assessed. At hatch, 1/3 of chicks received a single 10mL spray of ~8,000 GMT hyperimmune serum, 1/3 received two 10 mL sprays of the same hyperimmune serum, and the remaining 1/3 were left untreated. At three days of age, the three groups were divided in three again and one group was given an ocular-nasal dose of M41 strain, one group given an intramuscular dose of M41, and the final group left unchallenged. Respiratory signs were assessed at 4 DPC. Tears and blood were collected at 3, 6, and 13 DPC for viral load, IgA, and IgM assessment. Necropsies of ten birds per group were performed at 1-, 2-, 3-, and 4-weeks post challenge. At each necropsy swabs were taken for viral loads in the choanal cleft and rectum, tracheal cilia activity was assessed, and tracheas collected for histopathology.

RESULTS

Application method trial. Respiratory sign scores were significantly lower at 5 DPC in the spray application + challenge group compared to the challenge group alone (Figure 1). There were no significant differences in viral loads assessed at 5 and 10 DPC in either choanal clefts of cloacas. At necropsy, sinus and airsacculitis scores were lower in the spray + challenge group compared to the challenge group, significantly so for the airsacculitis scores. Tracheal epithelial thickness measures were unchanged between all challenge groups, however tracheal deciliation was significantly lower in the spray-treated challenge group compared to the untreated challenge group (Figure 2).

DISCUSSION

Spray application of hyperimmune serum showed promising results in terms of lowering acute respiratory signs and pathological effects, like incidence of airsacculitis and tracheal deciliation, of IBV challenge. This shows that the effects of maternal protection may be mimicked by supplemental delivery of IBV antibodies to chicks. It should be noted that, while these effects were observed, supplementation with serum antibodies did not lower the shed of the virus and did not totally mitigate the pathologic effects of challenge. For this reason, we hypothesized that it may be possible to heighten the protection offered by spray application of hyperimmune serum by increasing the dose of antibodies given. We also investigated if the affects of antibody supplementation seen in the first trial were simply due to blocking of the virus in the upper respiratory tract by observing the effects of challenge in the second trial either by ocular-nasal application or intramuscular injection. While not fool-proof, spray application of hyperimmune serum in commercial layer chicks may be useful in bringing maternal protection to uniform levels and extending the protection they provide during the first weeks of life. Further investigation will need to be done to assess the efficacy of spraying hyperimmune serum in commercial layer chicks already possessing natural maternal antibodies and to assess the effect of these exogenous antibodies in protection against long-term pathologies, like those seen in FLS, induced by early IBV challenge.

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Figure 1.

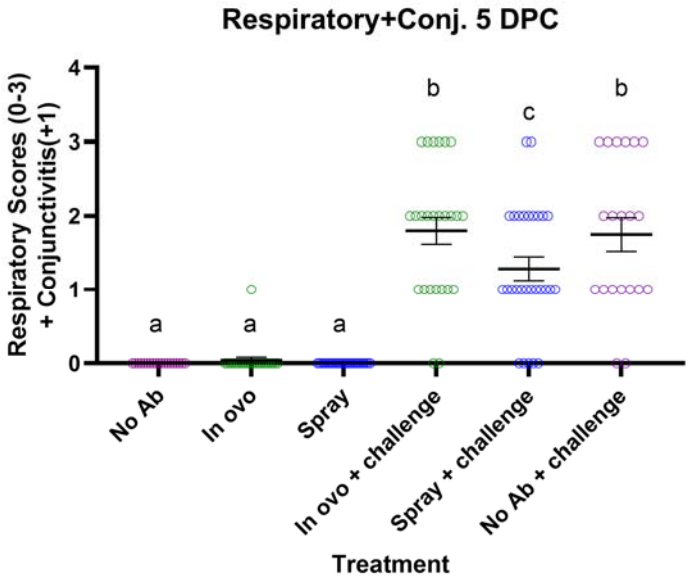
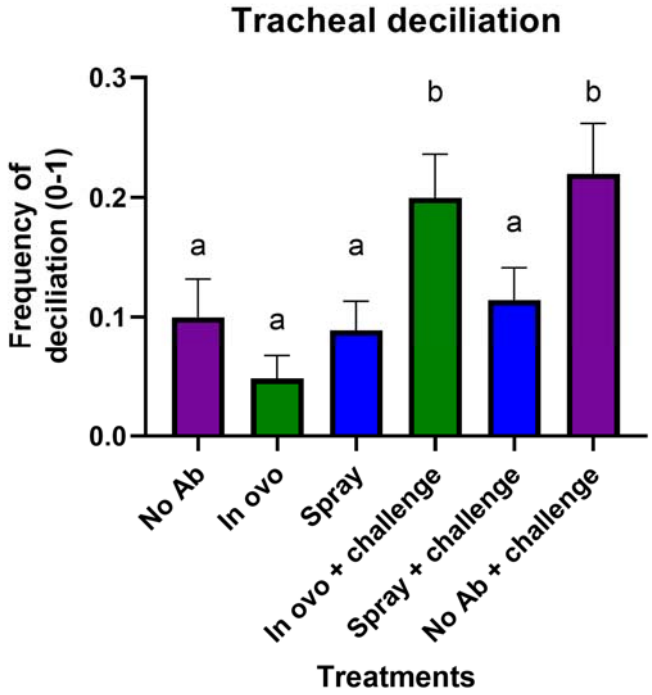


Figure 2.



COMPARATIVE TRANSCRIPTOMIC ANALYSIS OF CHICKEN-ORIGIN CELL LINES FOLLOWING AVIAN REOVIRUS INOCULATION

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INTRODUCTION

Avian reovirus (ARV) is a globally prevalent and rapidly evolving pathogen, responsible for significant economic losses to the poultry industry. The clinical presentation of the disease primarily involves viral arthritis and tenosynovitis. Additionally, frequent isolation of the virus from the gastrointestinal and respiratory tracts of both healthy and diseased birds has been demonstrated. Moreover, lesions in the liver, heart, and intestine, as well as a secondary induction of immunosuppression, have been associated with the virus.

Despite an expansive spectrum of pathological outcomes, the comparative cytopathogenicity and interplay of molecular mechanisms upon ARV infection, specifically across different cell-types, remain elusive. The aim of the present study was to describe an account of microscopic imaging and transcriptomic profiling to understand progression of infection *in vitro*.

MATERIALS AND METHODS

Chicken embryo liver (CELi) and kidney (CEK) cells were prepared using established methods. Chicken macrophage cell line HD-11 was kindly provided by Dr. Li Zhang, Mississippi State University, MS. Cells were grown in six well plates and inoculated with 10^2 $TCID_{50}$ ARV S1133. Five wells of each treatment as all as of an uninoculated control were evaluated as described below, and their supernatants were harvested for extraction of total RNA 6 hours post-inoculation (hpi), 12 hpi and 24 hpi.

Images were captured at 100X magnification from five random areas of each well without removing the medium. Using OpenCV2 computer vision module, thresholding of the images was performed. CPE areas were defined as large cellular aggregates appearing darker against the background. The count of CPE areas, their cumulative size and the ratio to the background was measured.

Supernatant was collected from the well and total RNA was extracted. Samples were submitted for whole transcriptome sequencing.

RESULTS AND DISCUSSION

For CELi and CEK cells, the cellular aggregations appeared distinctively darker than the cellular monolayer 24 and 48 hpi (Figure 2). The counts of CPE areas were significantly different between ARV infected and control groups (Figure 1). The ratio between CPE area and background differed also between infected and uninfected wells. While the method could distinguish between infected and uninfected monolayers effectively, certain limitations with precision were encountered while using the computer vision models. Due to the primary nature of the cells and apoptosis, a few artifacts were detected as counts of aggregation in the negative controls using thresholding methods. This is reflected in the non-zero counts of CPE areas in the negative controls.

For HD-11 cells, a different type of CPE (cell rounding) was observed and was not quantifiable using the threshold-based segmentation. Manual annotation of objects following training and validation of machine learning models could assist with more accurate detection and quantification of the CPE in microscopic images.

Transcriptome analysis revealed numerous differentially expressed genes and pathways between infected and uninfected cells depending on time point and cell type.

Automated recognition of CPE has the potential to yield more objective results than the traditional method. Together with the results of the transcriptome analysis, the results provide an insight into the reaction of various cells after infection with ARV.

(The full manuscript will be submitted to *Avian Diseases*.)

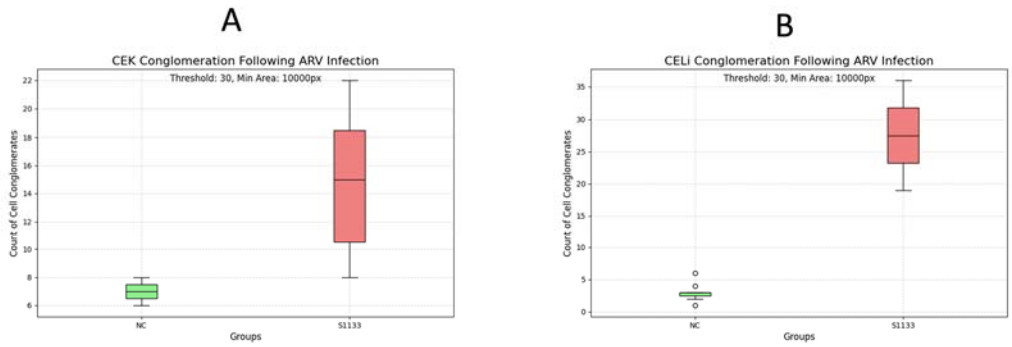

Figure 1: Chicken embryo liver cells (CELi) (A) and chicken embryo kidney cells (CEK) (B) were infected with Avian Reovirus (ARV). Areas with cell conglomerations indicating cytopathic effect (CPE) were counted based on thresholding.

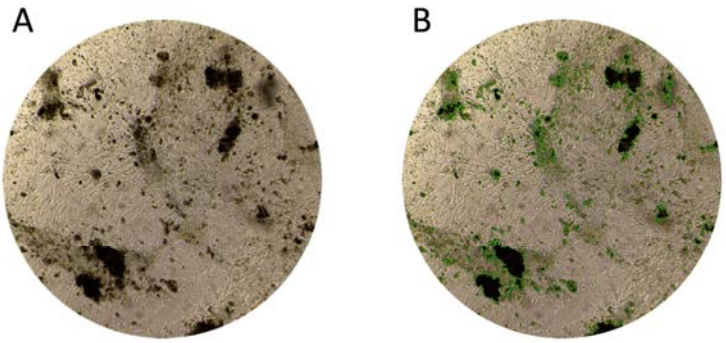

Figure 2: Chicken embryo kidney cells (CEK) infected with Avian Reovirus (ARV). Cell conglomerates indicated cytopathic effect (CPE) patterns (A). The areas were marked using OpenCV2 computer vision programming (B).

PROTECTION EFFICACY OF A RECOMBINANT HVT-ND-LT VACCINE WHEN ADMINISTERED INDIVIDUALLY OR IN COMBINATION WITH A TISSUE CULTURE ORIGIN (TCO) VACCINE AGAINST INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV)

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SUMMARY

Infectious laryngotracheitis (ILT) is a respiratory disease that causes significant economic losses to the poultry industry. Control of the disease is attained by vaccination and biosecurity. The use of bivalent and trivalent recombinant Herpesvirus of turkey (rHVT) vaccines expressing ILTV genes have increased worldwide. In the United States, vaccination programs of long-lived birds (broiler breeders and commercial layers) against ILT include immunizations with either HVT recombinant vector vaccines in the hatchery, or live attenuated vaccines administered via the drinking water [chicken embryo origin (CEO)] or eye-drop [tissue culture origin (TCO)]. The objective of this study was to evaluate the protection efficacy of a commercial trivalent rHVT-ND-LT when administered at one day of age followed by TCO vaccination via eye drop at 10 weeks of age. Groups vaccinated with rHVT-ND-LT, TCO, and the combination of rHVT-ND-LT + TCO were challenge with a virulent ILTV strain at 15 weeks of age. After challenge, clinical signs of the disease were significantly reduced in all the vaccinated groups. However, mortalities were only prevented in chickens vaccinated with the rHVT-ND-LT + TCO. To assess challenge virus transmission, contact naïve chickens were introduced to all vaccinated groups immediately after challenge. At 8 days post-introduction, infection of contact naïve chickens was evidenced in those introduced to the rHVT-ND-LT and TCO group, but prevented in the rHVT-ND-LT + TCO group. Overall, these results indicated that compared to rHVT-ND-LT or TCO when administered alone, the rHVT-ND-LT + TCO vaccination strategy improved protection against disease and reduced shedding of the challenge virus.

INTRODUCTION

Combinations of recombinant vector vaccines and live attenuated vaccines have been evaluated in search of safer and more effective ILT vaccination strategies. Previous results showed that rHVT-LT + CEO vaccination provided more robust protection than vaccination with HVT-LT alone, and priming with rHVT-LT reduced CEO virus replication, consequently buffering the CEO post-vaccination reaction (1). Also, when combining rHVT-LT with the ILTV gene-deleted strain (BΔORFC), administered in ovo or via spray at day of age, protection against ILT was improved (2). The objective of this study was to evaluate the protective efficacy of the trivalent rHVT-ND-LT recombinant and the live attenuated TCO vaccine when administered by themselves or sequentially in long-lived birds. Assessment of protection was based on clinical signs, challenge virus genome load in the trachea, histopathology scores, and viral transmission to contact naïve chickens.

MATERIALS AND METHODS

At one day of age (doa) a total of 107 SPF chickens were tagged in the neck and distributed into five different poultry colony houses. Two groups of 20 and one group of 19 chickens were vaccinated with the Innovax-ND-ILT® (rHVT-ND-LT) SC in the neck. Two groups of 24 chickens each were mock vaccinated with phosphate-buffered saline (PBS) in a similar fashion. At 10 week of age (woa) one group of rHVT-ND-LT vaccinated (n = 19) and one mock group (n = 24) were vaccinated with the TCO vaccine via eye drop and identified as rHVT-ND-LT + TCO and TCO, respectively. At 15 weeks of age the rHVT-ND-LT + TCO (n = 18, one bird was euthanized due to leg injury), rHVT-ND-LT (n = 20), TCO (n = 24), and non-vaccinated (NVx) (n = 24) groups were challenged (Ch) with the virulent strain 1874C5 at a

10^4 $TCID_{50}$dose delivered in a total volume of 200 µL/bird (50 µL per eye and 100 µL intratracheally). The remaining rHVT-ND-LT vaccinated group of chickens (n = 20) were mock challenged with tissue culture medium as described above and served as the negative control (rHVT-ND-LT/NCh). Clinical signs of the disease were evaluated from 3 to 7 days post-challenge (dpch) and tracheal swabs were collected at 3 and 5 dpch to evaluate challenge virus genome load by real-time PCR as previously described by Maekawa *et al*. (3). Immediately after challenge sets of five age matched SPF naïve chickens were introduced to each group of chickens (rHVT-ND-LT + TCO/Ch, rHVT-ND-LT/Ch, TCO/Ch, NVx/Ch, rHVT-ND-LT/NCh) to evaluate challenge virus transmission from challenge to naïve chickens. At eight days post introduction, clinical signs were evaluated in contact naïve chickens. Contact naïve chickens were then humanly euthanized, and the cranial segments of the trachea were collected for histopathological examination as previously described Guy *et al*. (4) whereas the adjacent trachea segment (3 cm) was collected for ILT genome load evaluation using real-time PCR (1). During the length of the experiment, chickens were given a standard feed diet, and water was provided ad libitum.

RESULTS

Clinical signs post challenge and survival of challenged chickens. The peak of clinical signs among all challenge groups was observed at 5 dpch. At 5 dpch, all challenged groups (rHVT-ND-LT/Ch, TCO/Ch, rHVT-ND-LT + TCO/Ch) showed a reduction in clinical signs compared to the positive control group (NVx/Ch). The median clinical signs score at 5 dpch for the TCO/Ch group was significantly lower than for the NVx/Ch group but not different than the rHVT-ND-LT/Ch or the rHVT-ND-LT + TCO/Ch group median clinical sign scores. In contrast, the median clinical signs score at 5 dpch for the rHVT-ND-LT + TCO/Ch group was significantly lower than the NVx/Ch and rHVT-ND-LT/Ch groups, but not different than the TCO/Ch or the negative control group. The rHVT-ND-LT + TCO/Ch group showed 100% survivability. Meanwhile, the TCO/Ch and rHVT-ND-LT/Ch showed 87% and 95% survivability, respectively, and 46% of the NVx/Ch group survived the challenge virus infection.

ILTV genome load in the trachea post challenge. The average genome load at three days post-challenge in the trachea of the rHVT-ND-LT/Ch group was not significantly different than the average viral genome load detected in the trachea of the NVx/Ch group. However, there was a significant reduction in the average trachea viral genome load for the TCO/Ch and rHVT-ND-LT + TCO/Ch groups of vaccinated chickens as compared to the NVx/Ch group. At five days post-challenge, the average trachea viral genome load of the TCO/Ch and rHVT-ND-LT + TCO/Ch groups were significantly lower than the average trachea viral genome of the rHVT-ND-LT/Ch and NVx/Ch groups. The average trachea challenge virus genome load of the rHVT-ND-LT + TCO/Ch was no statistically different than the negative control group.

Infection of naïve contact chickens. Naïve contact chickens were introduced to each group of chickens (rHVT-ND-LT/Ch, TCO/Ch, rHVT-ND-LT + TCO/Ch, NVx/Ch, rHVT-ND-LT/NCh) immediately after challenge. Median clinical signs scores for chickens introduced to the rHVT-ND-LT + TCO/Ch group were significantly lower than for chickens introduced to the NVx/Ch group. Median clinical sign scores for chickens introduced to the rHVT-ND-LT/Ch and TCO/Ch groups were not different from those of chickens introduced to the NVx/Ch group. The average viral genome load in the trachea of chickens introduced to the rHVT-ND-LT/Ch and TCO/Ch groups was similar to that of chickens introduced to the NVx/Ch group. However, viral genome load for chickens introduced to the rHVT-ND-LT + TCO/Ch group was significantly lower than for chickens introduced to the NVx/Ch group. Median lesion scores of the upper trachea for chickens introduced to the rHVT-ND-LT/Ch and TCO/Ch groups were not significantly different than the media trachea lesion score of chickens introduced to the NVx/Ch group. Compared to the median trachea lesion score of chickens introduced to the NVx/Ch, the median trachea lesion score for chickens introduced to the rHVT-ND-LT + TCO/Ch group was significantly lower.

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IMMUNE CELL PROFILE IN IMMUNE ORGANS OF BROILER CHICKENS FOLLOWING INFECTION WITH VARIANT INFECTIOUS BURSA DISEASE VIRUS SK09

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SUMMARY

In Canada, infectious bursa disease virus (IBDV) variant strains (varIBDV) are circulating. However, the detailed immune responses against varIBDV remain to be elucidated. In the current study, the percentage of immune system cells was analyzed in chickens with/without the maternal antibody (MaAb) against varIBDV after varIBDV infection using flow cytometry. An increase of $CD3\varepsilon^+$ T cells and monocytes/macrophages and a decrease of B cells were observed in bursa of Fabricius (BF) of MaAb-/challenged chickens at 5 days post-infection (dpi). $CD8\alpha\alpha^+$ γδ T cells was high in MaAb+/challenged chickens. B cell depletion was not observed in MaAb+/challenged chickens. After 14 dpi, B cell repopulation was partially observed in $MaAb^-$ challenged chickens. $MaAb^+$ challenged chickens had a high percentage of $CD8\alpha\alpha^+$ γδ T cells in spleen at 28 dpi. In conclusion, T cells, B cells, and macrophages may contribute to the pathogenesis of varIBDV, the protection from varIBDV, and the repopulation of the cells after infection.

INTRODUCTION

Infectious bursa disease virus (IBDV) is a highly contagious virus that affects young chickens. IBDVs are classified into two serotypes, serotype I and II (2). Serotype I IBDVs are pathogenic in chickens and virulent and very virulent strains cause high mortality in chickens while variant IBDV (varIBDV) leads severe immunosuppression (5). Therefore, IBDV is one of the major avian viruses causing significant economic losses in the poultry industry worldwide (2). In Saskatchewan, Canada, varIBDV, SK09, 10, 11 12, and 13, are circulating in the broiler chicken industry (8). IBDV infection starts mainly by fecal-oral transmission. After reaching intestine, the virus disseminates to bursa of Fabricius (BF) (9). IBDV targets B lymphocytes in the BF, specifically IgM^+ B cells, which can result in a significant reduction in antibody production and compromised immune function (11).

Our previous studies revealed that maternal antibodies (MaAbs) against SK09 can protect progenies from SK09 infection. However, the detailed mechanisms remain to be elucidated.

The aim of this project is to profile immune system cell subsets in intestine, the primary infection site, and in BF, the target organ by IBDV, in progenies with or without MaAbs against SK09 after SK09 infection using flow cytometry.

MATERIALS AND METHODS

Broiler breeders were vaccinated with live and inactivated varIBDV SK09 at 13 and 16 weeks of age, respectively. Eggs were collected from fully vaccinated breeders against varIBDV SK09 (MaAb+) or untreated breeders (MaAb-). Progenies with or without MaAbs against SK09 received 3×10^2 EID_{50} of varIBDV SK09 orally at day 6 of age (MaAb+/challenged or MaAb-/challenged group, respectively). MaAb free progenies without receiving SK09 were used as a control. Spleen and BF samples were obtained at 1, 5, 14, 21, 28, and 35 days post-infection (dpi) and single mononuclear cells were isolated using Histopaque1077. Cells were stained with antibodies to analyze T cell, gamma delta (γδ) T cell, B cell, and monocyte/macrophage (MoMa) subsets and their subpopulations. Stained cells were analyzed by CytoFLEX.

RESULTS AND DISCUSSION

$CD4^+$ T cell and $MoMa^+MHCII^+$ monocyte/macrophage populations were induced in BF of MaAb-/challenged chickens as early as 1 dpi. Macrophages have crucial roles in the initiation of immune responses in IBDV-infected chickens as they produce chemokines and cytokines to induce immune system cell migration to the infection site, inflammation, and activation of T cells and natural killer cells (7). Since $CD8\alpha^+$ T cells were dramatically induced in BF of MaAb-/challenged chickens after 5 dpi, the initial induction of $CD4^+$ T cells and macrophages may contribute to the activation of

cytotoxic T cells. In contrast, immunosuppressive activities of $CD4^+$ T cells and macrophages in IBDV-infected chickens have been suggested (7,10,13). They are believed to produce IL-10 and TGF-β which inhibit immune responses against IBDV. Due to the immunosuppressive milieu, varIBDV may not be eliminated immediately after invasion. γδ T cells play crucial roles in the initiation of immune responses as a first line of defense (4). In chickens, there are two subpopulations of $CD8^+$ γδ T cells, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ γδ T cells. It is reported that an increase of $CD8\alpha\alpha^+$ γδ T cells was observed in pathogen-infected chickens (3,6). In the current study, a significant increase was observed on both $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ γδ T cells in BF of MaAb-/challenged chickens at 5 dpi while $CD8\alpha\alpha^+$ γδ T cells were predominant in MaAb+/challenged chickens.

Further studies will be required to examine the functional differences between those γδ T cell subpopulations and their contribution to the varIBDV pathogenesis and the protection against varIBDV. A decrease of IgM expressing B cells was observed in BF of MaAb-/challenged chickens at 5 dpi but not in MaAb+/challenged chickens compared to the control group. IgM^+ B cell subpopulation gradually recovered in BF of MaAb-/challenged chickens after the B cell depletion at 5 dpi. The frequency of IgY^+ B cells in BF of the MaAb-/challenged group was significantly higher than that in the control group after 14 dpi.

These results indicate that a viral reduction by antibodies against varIBDV in the early infection phase is a key to prevent B cell depletion. Progenies are protected from pathogens by MaAbs until 2–3 weeks of age. However, chickens become susceptible to IBDV infection after the period (1). A previous IBDV study revealed IBDV-infected macrophages in spleen of chickens with high MaAbs against IBDV and suggested the possibility of IBDV replication in BF after the decline of the MaAB level (12).

In the present study, although B cell population was not decreased in MaAb+/challenged chickens through the experimental period, a decrease of $MoMa^+MHCII^+$ cells and an increase of $CD8\alpha\alpha^+$ γδ T cells were observed in spleen of MaAb+/challenged chickens at 28 dpi. These results suggest that varIBDV reactivated in spleen at 28 dpi but was eliminated by immune system cells immediately after reactivation. In conclusion, MaAb-/challenged chickens showed the early induction of $CD4^+$ T cell and $MoMa^+MHCII^+$ cells in BF, and subsequently $CD8\alpha^+$ T cells and IgY^+ B cells were induced after 5 dpi and 14 dpi, respectively. MaAb+/challenged chickens showed a higher percentage of $CD8\alpha\alpha^+$ γδ T cells and a lower percentage of $MoMa^+MHCII^+$ cells in spleen at 28 dpi. These immune system cells may contribute to both the pathogenesis of varIBDV and the protection from varIBDV in broiler chickens.

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EFFICACY OF AN HVT-ND-IBD AND LIVE ATTENUATED VACCINES AGAINST FIELD TWO IBD VARIANTS AFFECTING PERFORMANCE IN COMMERCIAL BROILERS IN THE UNITED STATES

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INTRODUCTION

Infectious bursal disease (IBD) is a viral disease widely disseminated in poultry that induces dysfunction of the immune system. Birds suffering from this disease are immunosuppressed, more susceptible to opportunistic infections, develop a poor response to vaccinations and reduction in overall performance. Even though classic and variant IBD strains have been isolated in the United States (US), variant strains are more prevalent. The most effective way to control this disease is by vaccination. Currently, recombinant vaccines are becoming widely adopted because they can be applied in ovo or subcutaneously at one day of age (DOA) alone or in combination with live attenuated vaccines.

Recently, two IBD variant strains were isolated from broiler flocks affected between 20 and 30 days of age and exhibiting increased feed conversion and bursal atrophy. Specific Pathogen Free (SPF) birds were placed in isolation units and challenged at 26 days of age. The field isolates were characterized as Delaware E-like and T1 variant strains.

The objective of the study was to assess protection against these isolates by using a dual recombinant rHVT-ND-IBD vaccine, alone or in combination with the 89/03 strain, or the ST-12/Del 51A combined live attenuated vaccine.

Herein we report a process for analyzing the different vaccine combinations efficacy and field challenge for IBD performed in environmentally controlled isolation units at the University of Georgia facilities located in Athens, Georgia, United States.

MATERIALS AND METHODS

Specific Pathogen Free (SPF) birds used in this study were placed in negative pressure isolation units with unrestricted access to feed and water. The challenge virus used in this study was the variant DMV4952-07 strain, isolated from field samples from affected flocks submitted to the Poultry Disease Research Center at the University of Georgia.

The challenge viruses were expanded in 3-week-old SPF chickens, titrated in chicken embryos, and diluted in tryptose phosphate broth (TPB). Birds in groups 1-10 were challenged by eyedrop with $10^{3.0}$EID50/dose for IBDV variant DMV4952-07 and $10^{2.5}$ EID50/dose for IBDV variant T1. Two hundred and twenty (SPF) birds were divided into eleven treatment groups as follows:

1. rHVT ND-IBD challenged with IBDV T1
2. rHVT ND-IBD challenged with DMV4952-07
3. rHVT ND-IBD + 89/03 challenged with IBDV T1
4. rHVT ND-IBD + 89/03 challenged with DMV4952-07
5. rHVT ND-IBD + ST-12/Del 51A challenged with IBDV T1
6. rHVT ND-IBD + ST-12/Del 51A challenged with DMV4952-07
7. rHVT ND-IBD + 89/03 + ST-12/Del 51A challenged with IBDV T1
8. rHVT ND-IBD + 89/03 + ST-12/Del 51A challenged with DMV4952-07
9. Non-vaccinated/Challenged with IBDV T1
10. Non-vaccinated/Challenged with DMV4952-07
11. Non-vaccinated/Non-Challenged

At 26 days of age, 10 birds in each group were bled for IBDV XR ELISA (IDEXX). Five birds per group were euthanized and body and bursa weights collected. Bursas were fixed in neutral buffered formalin and submitted for histopathology and scoring. The challenged groups for the respective variant strain were placed in separate rooms.

Each bird in groups 1-10 were challenged by the intraocular route of inoculation with 0.05 mL of the respective challenge virus. Five birds from each group were euthanized at 26 days-of-age (day of challenge). The study was terminated at 33 days of age (seven days post challenge). Individual bird body and bursa

weights were measured and bursas collected in neutral buffered formalin for histopathology scoring.

DISCUSSION

Protection provided against DMV4952-07 and T1 IBDV variant strains using a dual recombinant HVT-ND-IBD vaccine (rHVT-ND-IBD) alone or in combination with the 89/03 strain (variant attenuated), or the ST-12/Del 51A combined live attenuated vaccine (classic intermediate strain) was evaluated.

The mean bursa/body weight ratios of all vaccinated and challenged groups at 33 days of age (seven days post challenge) were significantly higher than unvaccinated/lBDV variant Tl and DMV4952-07 challenge control groups demonstrating complete protection against bursa.

No significant differences in bursa/body weight ratios and bursal lesion scores were observed between the vaccinated groups at 33 days of age (7 days post challenge). This demonstrates that all vaccine combinations provided complete protection against bursa atrophy following challenge with IBDV variant Tl and DMV4952 07 field isolates. The bursa/body weight ratios for all vaccinated/lBDV variant challenge groups were higher than the bursa/body weight protection score demonstrating good protection against bursal atrophy following challenge using this parameter of evaluation. No significant differences in bursa/body weight ratios were observed between vaccinated/lBDV variant Tl challenged groups and the unvaccinated/unchallenged group demonstrating excellent protection in all vaccinated/challenged groups.

In conclusion, the dual recombinant HVT-ND-IBD vaccine (rHVT-ND-IBD) alone or in combination with the 89/03 strain (variant attenuated), or the ST-12/Del 51A combined live attenuated vaccine (classic intermediate strain) provided complete protection against challenge with IBDV variant field isolates Tl and DMV4952-07.

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IMPROVEMENTS ON THE WATERFOWL ALERT NETWORK

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Two major outbreaks of highly pathogenic avian influenza (HPAI) in North America (2014-15 and 2022-current) have led to the depopulation of over 100 million domestic poultry with a total economic impact of over $6 billion dollars and counting. Since waterfowl are the primary reservoir of avian influenza viruses, understanding how waterfowl abundance/presence changes over space and time is an essential component of poultry husbandry, logistics and biosecurity. The WaterFowl Alert Network (WFAN) represents the world's first daily predicative tool to predict waterfowl distributions on a daily basis at fine spatial resolution. The tool is currently operating in 20 states in the U.S. with the potential to expand on multiple continents. Here we will provide updates on the technology including: risk scoring, 3-day forecasting, geo-fencing, comparison between telemetry and radar modeling and analysis of data between affected farms, physical biosecurity, operational biosecurity and waterfowl abundance.

From a practical perspective, to date, no research efforts have been performed to comprehensively model both on-farm and off-farm risk factors for HPAI to determine which factors or combination of factors are truly causal for HPAI outbreaks. While broad based biosecurity measures have been effective in shifting HPAI risk patterns – in the 2014-2015 outbreak, 70% of HPAI cases were attributed to lateral spread (from farm to farm), while in the latest outbreak lateral spread has been reduced to 15%. Therefore it is clear that these on-farm recommendations and practices have limitations. A more comprehensive analysis of both on-farm *and* off-farm risk factors could provide additional insights and recommendations beyond those for basic biosecurity.

During the spring of 2023, USDA released their 2022 USDA-CEAH Interim Action Report on HPAI[24] transmission. Among other results, the report noted "intense periods of bird migration as seen by BirdCast maps that correlated with outbreaks in domestic poultry." The report also stated that "…this tool can be used to raise awareness of increased HPAI risk due to wild bird movements." The report also cited eBird data, noting that "HPAI positive premises were more likely to be detected within the first seven days of heavy wild bird observation with a 50 km spatial window." While the utilization of BirdCast and eBird data may be attractive conceptually, it should be noted that the 50 km observational window employed is likely too coarse for use as an effective prediction, analysis, and risk analysis tool. Within areas of high commercial farm density, hundreds of farms can be located within such a large analysis window. *However, both this action report and the USDA case-control risk factor study mentioned above demonstrate the growing interest in understanding waterfowl abundance and/or waterfowl occupancy as potential risk factors in addition to using tools that are more spatially precise such as the WFAN.* A review of various approaches will be summarized in addition to presenting data related to on and off-farm risk factors for affected and unaffected farms during the current outbreak.

INCORPORATING VACCINATION AND GAME FOWL INTO A NEWCASTLE DISEASE MODEL

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SUMMARY

Large-scale Newcastle disease (ND) outbreaks such as those in 2002 and 2018-2020 represent a large threat to the California poultry industry as well as backyard bird owners. To better prepare for future outbreaks, USDA's Center for Epidemiology and Animal Health constructed a model for the spread of ND in California and the surrounding states based on the 2018-2019 ND outbreak in California, focusing primarily on transmission of ND among commercial and backyard premises. The USDA model is a stochastic spatial model, and the primary mechanism of the model is to simulate the movement of birds, as well as other types of movements able to spread ND virus such as fomite transmission and local area spread. Farm locations in the model are simulated by the Farm Location and Animal Population Simulator tool, and the commercial premises are broken down by size and type, with unique parameters for each. In addition to chickens, these premises are also able to incorporate turkeys and specialty birds. In addition to disease spread, the model incorporates detection and depopulation times, based on probability distributions unique to each class of farm, typically based on size and whether the premise is commercial or not. The ND baseline model assumed a completely naïve population with regards to immunization, as vaccination levels among backyard premises are typically low. Our work focuses on updating the model to include two additional features that are potentially relevant to ND transmission patterns: game fowl and vaccination.

The inclusion of game fowl in the model is vital, as the three most recent large-scale ND outbreaks in California (i.e. 1971-1974, 2002-2003 and 2018-2020) involved game fowl to some degree, making the movement patterns of game fowl communities critical to understanding ND outbreaks. To include these movements, we have aimed to implement distinct movement patterns for game fowl owners who take their birds to shows and fairs and those who participate in derbies, as well as those who may participate in both. These movements are designed to depict the temporal patterns and distance of movements of birds to the gatherings, as well as to capture the transmission risk for both direct and indirect contacts that occur at derbies and shows. The distribution of game fowl is informed by data collected by collecting data from marketplaces such as Craigslist on game bird sales as well as survey data.

Including vaccination in the model allows us to investigate how different levels of vaccination affect ND transmission dynamics. Vaccination is of interest because it is effective at reducing mortality and viral shedding rates when performed correctly, but also has the capacity to mask clinical signs in infected, vaccinated birds. Currently, we are focused on exploring the effects of individuals electing to vaccinate birds, primarily game fowl owners, but future work will examine the effects of vaccination campaigns in response to outbreaks as well. Implementation of vaccination in the model requires the incorporation of game fowl as well, as this population is the one most likely to vaccinate and the one with its transmission patterns most affected by reduced viral shedding but also the possibility of silent transmission. Our model incorporates the high levels of incorrect vaccine usage often seen with ND in non-commercial premises and allows for experimentation with both different levels of vaccination and different rates of correct vaccine usage. Experimenting with different levels of vaccination and correct usage, especially among game fowl, allows us to evaluate the impacts that vaccination has on ND virus transmission through the game fowl community, as we incorporate the reduction in infectivity to represent reduced shedding and the reduction in mortality, but also incorporate silent transmission by reducing the probability of detection for correctly vaccinated flocks. We do not offer a verdict on the utility of vaccines, instead we only report the findings of the model we have built based on the data available to us.

Here we present results from the implementation of these model features into the ND baseline model, focusing primarily on the role game fowl premises play in viral transmission dynamics and the effects of different rates of vaccination on duration and severity of the simulated outbreaks. Further work on this project will investigate the economic impact of outbreaks under different sets of conditions, and the effects of certain policies and response measures.

SURVIVING HIGHLY PATHOGENIC AVIAN INFLUENZA: PERSPECTIVE OF AN ANIMAL SANCTUARY'S RESPONSE TO A DEADLY VIRUS

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SUMMARY

In February 2023, the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) confirmed a detection of highly pathogenic avian influenza (HPAI) at Harvest Home Animal Sanctuary, a non-profit organization operating in California's San Joaquin County. Since 2004, the sanctuary has provided shelter and care for a variety of species at its two acre property located on the San Joaquin Delta, an area known for its abundance of wildlife in a rural agricultural setting.

In reference of HPAI, Harvest Home Animal Sanctuary was categorized a "WOAH Non-Poultry", a designation established by World Organisation for Animal Health (OIE). The designation identifies locations where non-commercial backyard flocks reside. Chickens, turkeys, ducks, geese, pigeons, and doves were among the domestic bird species housed at the sanctuary at the time of the virus detection. As a "WOAH Non-Poultry" site, the sanctuary was afforded an option to complete a 120-day government HPAI quarantine without a mandatory depopulation intervention. The sanctuary elected to complete the quarantine without depopulation option from February 2023 to June 2023.

At the beginning of the quarantine, the mortality among the sanctuary's chicken and turkey population was projected to be ninety to one-hundred percent. When the sanctuary was released from its quarantine on June 16, 2023, a twenty percent mortality was recorded. This narrative details the response plan the sanctuary employed to navigate through the HPAI outbreak at its location.

INTRODUCTION

Harvest Home Animal Sanctuary housed bird species in nine separated areas at its two acre location. On the morning of February 14, 2023, an employee of Harvest Home Animal Sanctuary discovered the remains of three birds (one turkey tom and two chicken hens) in one of the two "Main Yard Barns". The employee immediately alerted her findings to the on-site supervisor. In the days prior, there were no observed clinical signs of illness present in the deceased birds. When the on-site supervisor arrived to inspect the birds' remains, signs of lethargy and loss of appetite were noted among the live chickens and ducks housed in the barn.

Based on the sudden bird mortality and noted clinical signs in the living birds, an immediate decision was made to submit the remains of the turkey and two chickens for necropsy to the California Animal Health & Food Safety Laboratory in Turlock, California on February 14th. During the evening of February 15th, 2023, the sanctuary was notified of the presumptive HPAI positive finding by the assigned case investigator from the California Department of Food and Agriculture (CDFA).

MATERIALS AND METHODS

With the confirmation of the presumptive HPAI positive result, two field veterinarians with the California Department of Food and Agriculture scheduled the initial site inspection at Harvest Home Animal Sanctuary on February 16th, 2023. The parameters of a 120-day government "quarantine without depopulation" was shared in detail. At the meeting, the sanctuary selected this option.

At the time of the HPAI detection, the sanctuary had nine areas on its premises that housed domestic birds, including chickens, turkeys, ducks, geese, pigeons, and doves. All the birds on the property were considered exposed to HPAI. However, morbidity and mortality was only observed in three areas – located at the back of the property. Each of the three affect housing areas were approximately twelve feet in proximity to each other.

As the quarantine was established, each of the sanctuary's nine bird areas sheltered-in-place to curb the spread of HPAI. One sanctuary employee (i.e. on-site supervisor) was designated to work exclusively in the affected bird areas while the additional employees were assigned to work in the unaffected bird areas along with the areas that housed mammals, including pigs and goats. A relief person was available to function as back-up support in the affected bird areas as needed. Documentation through videos and

photography of the unaffected areas was shared by assigned personnel with the on-site supervisor to monitor any new clinical signs of HPAI. During the course of the quarantine, no additional clinical signs of HPAI developed in the six areas on the premises. In the affected areas, biosecurity items for personnel included disposable coveralls, gloves, masks, hairnets, and foot booties. In the unaffected areas, however, biosecurity items included disposable gloves, masks, and foot booties.

RESULTS DISCUSSION

On June 16th, 2023, Harvest Home Animal Sanctuary was released from its HPAI government quarantine. At the conclusion of the quarantine, one wave of mortality was attributed to HPAI. From February 14th through March 2nd, 26 bird deaths were documented. Thereafter, there was a period of approximately 40 days without a single bird death. During the remaining time of the quarantine, several unrelated bird deaths were documented. HPAI was not presumed to be the cause of death. The sanctuary's HPAI response plan was influenced by the following factors:

- Rapid Virus Detection
- Heighten Biosecurity Practices
- Balancing Overall Bird Wellbeing (High Quality Nutrition and Bird Enrichment)

Of the 160 birds on the sanctuary premises, twenty percent bird mortality was documented at the sanctuary during the quarantine period. The projection of ninety to one-hundred percent mortality among chickens and turkeys was not the realized outcome at the sanctuary. Further investigation of the HPAI "quarantine without depopulation" option is worthy of consideration in the future to determine if lower mortality is observed at other "WOAH Non-Poultry" locations where HPAI is detected.

ACKNOWLEDGEMENT

Navigating through Harvest Home Animal Sanctuary's HPAI experience would not have been possible without the compassionate guidance and support of the following veterinarians:

- Jennifer Brinson, DVM
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- Holly Galusha, DVM
- Tino Luehman, DVM
- Brian Speer, DVM, DIP ABVP, ECZM

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This narrative is based on ob servations and recordkeeping collected by Harvest Home Animal Sanctuary along with written and verbal guidance provided by the California Department of Food and Agriculture.

Table 1. HPAI Bird Mortality at Harvest Home Animal Sanctuary

Date	Mortality	Affected Bird Species
2/14/23	6	Chicken, Turkey
2/15/23	2	Chicken
2/16/23	4	Chicken
2/17/23	1	Chicken
2/18/23	2	Goose, Duck
2/19/23	0	-
2/20/23	2	Goose, Turkey
2/21/23	1	Turkey
2/22/23	3	Chicken
2/23/23	1	Chicken
2/24/23	2	Chicken, Duck
2/25/23	0	-
2/26/23	1	Duck
2/27/23	0	-
2/28/23	0	-
3/1/23	0	-
3/2/23	1	Duck
	26	

Image 1.

Re: Quarantine: MO-HPAI-2022.101

Attachment C: Required Practices for HPAI Backyard Premises
(Quarantine without Depopulation)

Quarantined backyard premises without depopulation will be required to adhere to restrictions and conditions including but not limited to the following:

1. Premises must follow biosecurity practices.
2. Continue to provide food, water, and shelter for birds on the premises. Make sure wild birds cannot access feed and water sources.
3. The premises owner must ensure disposal of any eggs and bird carcasses daily in a biosecure manner. (Double bag in 3mil (contractor grade) black trash bags. Spray the outside of each bag with a disinfectant that is effective for Avian Influenza, such as Lysol. Securely close the bag and place in trash or take to landfill.)
4. No visiting other bird premises. Use dedicated footwear for working with or around birds. Clean and disinfect footwear, vehicle and vehicle tires, and equipment before leaving premises.
5. Notify Incident Management Team (IMT) of any dead birds.
6. Unannounced premises compliance checks may occur at any time.
7. The IMT may revise these conditions at any time based on disease events and risk

INFECTIOUS BRONCHITIS REAL-TIME-PCR ASSESSMENTS IN BROILERS

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INTRODUCTION

Infectious bronchitis (IB) is the most common viral respiratory disease in the broiler industry in the USA. The need to generate consistent and reliable data for the monitoring of vaccine take and field infections is critical to ensure that vaccination strategies are meeting the expectations in terms of proper vaccine administration and coverage, keeping track of circulating field virus (1,2). High loads of wild-type IBV strains combine with vaccine viruses creates the perfect scenario for variant virus generation (3).

Realtime PCR information has become a great diagnostic tool to help control infectious bronchitis. It is possible to generate data for each type of intervention and an analytical process that allows production managers to analyze, interpret and extrapolate the results. It is fundamental for coronavirus epidemiology to determine first the levels of virus circulation. The genetic variability can be done by means of Next Generation Sequencing (NGS) or Sanger sequencing results, a two-step complementary epidemiological assessment for broiler flocks that could help production managers fine tune interventions.

MATERIAL AND METHODS

Monitoring objectives for broiler flocks:

1. Monitoring hatchery vaccination: define vaccine take by measuring the amount and range of vaccine virus present in the upper respiratory tract at 4-6 days of age in a representative set of samples (15 birds/flock) using a consistent sampling protocol, at least six times per year.
2. Monitoring the field virus interaction with the flock immunity, vaccine efficacy and protectotype coverage between 28-35 days of age, at slaughter or at the season where an increase in clinical respiratory cases is expected.
3. Custom monitoring according to the production management system and field conditions: such as *E.coli* epidemiology (4,5).

The sampling protocol is defined according to the objective (6,7,8):

1. Number of samples: higher to build the baseline; this will increase the external value of the reference range.
2. Pooling criteria: 3-5 samples; pooling is not recommended for hatchery vaccine take evaluations.
3. Ideal and consistent swab type: Flocked swab/synthetic (rayon/nylon) (9), mini-tipped small head-swab for chicks (4-6 days old) or regular-size swabs for older birds.
4. Organ to sample: for example, choanal cleft.
5. When, where and why according with the monitoring objective and baseline dynamics.
6. How often: Complex size, number of processes flocks per week/month.

Samples handling, transport, transfer, and storage:

1. Type of media and consistency of the same media (not always needed if only for RT-PCR).
2. Cooling system and temperature range.
3. Define maximum time in hours to transport/transfer and processing the samples.

Sample extraction

1. Magnetic beads are ideal for RNA targets
2. Most important is the consistency of using always the same extraction method and same protocol that has being validated in each laboratory with internal panels.

Amplicon consistency:

1. Positive control range
2. Negative control validity
3. Possible role of PCR inhibitors
4. Company panels (references)

DISCUSSION

The key starting point of the validity and trustworthiness of reference data relies on how a baseline has been developed. This includes antigen thresholds, vaccine/field virus interactions and accuracy of the monitoring final interpretations. The information used for the generation of a baseline should include clinical data, pathology and epidemiology information of IB, including aspects

related to its pathogenicity, antigenicity, infectivity, prevalence and potential *E.coli* interactions. Data analytic principles could help guide to develop better understanding and know-how of the process.

It is not just about detecting IB virus-RNA but to develop and establish reference of cycle thresholds (CT values) (10,11), increase epidemiological knowledge and interpretations to enable more interventions based on information. These thresholds reference could help improve the use of information coming from Next Generation Sequencing or similar technologies.

Consistency could be achieved by standardizing sampling protocols (12), standardizing laboratory practices, aiming best possible quality control and quality assurance in sample handling and standardizing the mapping workflow and processes.

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INVESTIGATION OF EPIDEMIOLOGICAL BEHAVIOR OF 2016-2022 INFECTIOUS CORYZA OUTBREAKS IN CALIFORNIA

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SUMMARY

Avibacterium paragallinarum (AP), the bacterium responsible for infectious coryza (IC), has gained more attention over the recent years due to its increased prevalence in California and its economic significance in both the broiler and layer industry. Increased numbers of positive IC cases were seen in the California Animal Health and Food Safety Laboratory (CAHFS) system in 2017. This paper analyzes broiler, layer, and backyard poultry submissions that were positive for IC in the CAHFS database from 2016-2022 in order to assess the epidemiological behavior of this disease prior and after the initial 2017 outbreak. The results demonstrate that poultry type and location had a statistically significant impact on the likelihood of being IC positive.

MATERIALS AND METHODS

Case selection and definition. The CAHFS database was used for the selection of cases. A case was defined as any accession from January 1st, 2016 through December 31st, 2022 that contained chicken(s) with gross findings consistent with IC and were then positive for IC via isolation and identification of *Avibacterium paragallinarum* and/or polymerase chain reaction (PCR).

Case categorization. Cases were organized by the date of submission, age, poultry type (ie. backyard poultry, commercial broilers, commercial layers), and county. The date of submission was then used to categorize the cases into seasons with the following definitions for each season: winter (December-February), spring (March-May), summer (June-August), and fall (September-November). The counties were also divided into Northern California, Central California, and Southern California. Age was divided into juvenile and adult birds, with juvenile birds being less than five months old.

Isolation and identification. Swab samples were mainly collected from the sinus and trachea, but a minority of samples were collected from the coelomic cavity, lungs, conjunctiva, nasal cavity, respiratory exudate, pericardium, oropharynx, and air sacs. These samples were then cultured on MacConkey agar and 5% sheep blood agar and chocolate blood agar (CHOC). Both plates were cross-streaked with *Staphylococcus aureus* ATCC 25923 and then incubated at 37°C and 7% CO_2 for 48 hours (1). Colonies with a morphology consistent with *Avibacterium paragallinarum* were then confirmed via PCR using previously described techniques (2).

PCR. For cases found positive via PCR alone, samples from the sinus and trachea were collected and placed in a phosphate buffer solution (PBS). Previously described techniques were then used to detect *Avibacterium paragallinarum* via real-time PCR using a primer probe assay (3, 4).

Statistical analysis. A univariable logistic regression model was performed to test the association between IC positive cases and age, poultry type, location, and seasonality. Those with a statistically significant association then underwent a multivariable logistic regression model (5). A forward stepwise variable selection was used to add the variable with the lowest p-value to construct a final model with a significant level of $p \leq .05$. The models were compared with previous ones using a likelihood-ratio test after the addition of each variable. Model fit was evaluated using the Hosmer-Lemeshow test. Odds ratios (ORs), 95 % confidence intervals (Cls), and p-values were estimated using maximum likelihood methods. Analysis was performed using SPSS Statistics 29.0 (IBM, USA).

RESULTS AND DISCUSSION

Raw data trends. The average age of affected commercial broilers was 37.6 days with a standard deviation of 10.1 days, and for affected commercial layers, the average age was 41.8 weeks with a standard deviation of 24.6 weeks. The majority of the positive cases evaluated were from backyard poultry (51.0%, n = 346), and they mainly originated from Los Angeles County (n = 40), San Joaquin County (n = 26), and Riverside County (n = 25). Commercial layers made up 31.2% (n = 212) of the positive IC submissions and were mainly localized to Merced County (n = 72) and Stanislaus County (n = 50). Commercial broilers made up the least number of cases (17.8%, n = 121) and were

localized mainly to Merced County (n = 100). When evaluating seasonality, the majority of the cases analyzed occurred during spring (32.99%) followed by summer (29.4%), fall (21.06%), and then winter (16.49%).

Overall, there has been a general upward trend in testing in all three poultry types. This is best shown in 2018, when 352 IC tests were performed on backyard birds with only 91 (25.9%) being positive for IC.

Statistical analysis. Age, poultry type, spatial distribution, and seasonality were evaluated in the univariate regression analysis in which poultry type, age, and location were significantly associated with IC ($p < .05$). The final model showed poultry type and location as the only significantly associated factors with infectious coryza.

Layer birds were 1.3 times more likely to have IC than backyard poultry (95% Cl 1.01-1.65, OR = 1.3, $p = 0.039$); in contrast, broilers were less likely to have IC than both backyard poultry and layers (95% Cl 0.24-0.42, OR 0.31, $p < .001$). An important reservoir for IC transmission is asymptomatic carriers, and therefore, layers are likely more at risk for IC than backyard poultry because they have a higher density of birds, and unlike broiler production systems, layers are often in multi-age facilities that allow for the existence of more asymptomatic carriers (1, 6, 7).

When analyzing location, Central Valley (95% Cl 1.50-2.73, OR = 2.00, $p < 0.001$) and Southern California (95% Cl 1.48-2.79, OR = 2.00, $p < 0.001$) were two times more likely to have IC than Northern California. Central Valley contains the top poultry production counties within California; in contrast, a 2013 retrospective study examined all backyard poultry CAHFS submissions from 2007 through 2012 and showed that the county with the most backyard poultry submissions was Los Angeles County (8, 9). Therefore, it is reasonable that Central Valley and Southern California have more IC positive cases as they are places in which poultry are more prevalent.

Overall, the information in this study can be used to increase surveillance in chickens that are considered higher risk for IC (ie. commercial layers and birds located in Central Valley and Southern California). Uninvestigated variables in this study that include but are not limited to vaccination history, litter management practices, and biosecurity practices can be future avenues for research. Subsequent studies can focus on cases that include collection of this type of information at the time of submission and/or biosecurity surveys of commercial and backyard operations that are IC positive. This can help contribute to an even better understanding of birds at risk for IC.

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

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CRACKING COMPLEX CASES WITH EGGSPERT INSIGHTS AND DIAGNOSTIC TOOLS

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SUMMARY

This manuscript will work through a complicated clinical case with the reader. Case history will be reviewed, differential diagnoses assembled, and diagnostic tests submitted will be reported. The reader/audience will systematically and chronologically step through the testing results and arrive at the final diagnosis. The oral presentation associated with this case report will also provide an overview of new or novel diagnostic techniques, currently in play or in development. A short "back to basics" review of some simple necropsy and diagnostic techniques that are sometimes overshadowed by newer diagnostic methods, and which are still important tools to keep in mind will also be covered. These latter two parts of the presentation will unfold in the context of clinical cases where these tools have been used and are not covered in this manuscript.

INTRODUCTION

Population medicine, and the diseases that may circulate in populations, continue to persist and even evolve. The potential for multiple co-morbidities, which together ultimately result in the clinical signs and pathology that we observe, can be difficult to accurately capture and/or diagnose. For this reason, reviewing and learning from other complex cases of a similar nature is of continued importance to a successful and accurate diagnosis and to the prevention of future complicated cases. Not all cases have a singular diagnosis. And many disease presentations that we face today are the result of an unintentional miss-step, miss-management or a mistake; something else that opened the door and allowed 'disease' to enter. This case report aims to set the stage for the reader to identify some of the 'misses' that resulted in the clinical presentation described and to summarize the diagnostic steps taken to arrive at a diagnosis.

CASE REPORT/CASE PRESENTATION

Signalment and history. In 2023, on a Friday, a grower in Northeast Georgia reported a "cough" in a flock of 28-week-old broiler breeders (Ross 708 X YPM). The flock had achieved 10% egg production at 25 weeks of age (5% above breed standard) and was at 58% egg production (12% below breed standard) at the time of the initial complaint. Hen body weights were on target relative to breed standard at 25 weeks of age, and were slightly more than a half-pound (300g) above standard at the time of the complaint. Four days following the initial complaint, the flock experienced increasing mortality and the grower reported trembling birds. When mortality reached 0.23% in a single day, birds were submitted for necropsy with a request to test for *Mycoplasma* and infectious laryngotracheitis virus (ILTV) and two days later a field visit was conducted.

Examination findings/necropsy findings. Initially five live birds were submitted to the Poultry Diagnostic and Research Center (PDRC) for examination and sample collection. Two of the five birds were not in egg production. There were no other notable necropsy findings in the submitted birds. Samples were collected for bacteriology, histopathology and molecular testing for *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS), as well as ILTV by PCR.

During the field visit two days later, morning feeding was observed and two birds were discovered panting, exhibiting muscle tremors and unable to rise. These two birds were blood sampled and then euthanized for necropsy in addition to nine dead hens also collected during the course of the visit. Seven, of the 11 birds examined, exhibited hyperemic follicles and had a shelled-egg in the reproductive tract. Five of the examined birds had edematous to fibrinous fluid present between the pectoralis major and minor muscles (Figure 1). Seven birds were over-fleshed (breast muscle protruding up over the keel), two birds were not in production, one bird had peritonitis/ceolomitis, and six birds had cecal

worms. It was also noted during the visit that eggs appeared paler than anticipated and some birds exhibited a cough or snick. Egg production, as previously mentioned, was below breed standard.

In the context of the flock history, clinical signs observed and the necropsy findings, multiple differential diagnoses were considered for the flock. The primary differentials considered for birds found panting, trembling, immobile, and/or dead with intermuscular edema, were non-infectious; calcium tetany, ionophore toxicity, and "other" toxicity. Differential diagnoses considered for respiratory signs, reduced egg production and production of pale eggs included both infectious (IBV, NDV, low pathogenicity AI, and egg drop syndrome) and non-infectious etiologies (nicarbazin exposure, stress, and sulfa medication).

Based on these differentials, tracheal samples were collected to confirm the flock was negative for avian influenza (AI) and Newcastle disease virus (NDV), as well as to evaluate for infectious bronchitis virus (IBV). Additional tissues including trachea, skeletal muscle, heart, and gizzard were collected for histologic examination. The feed mill manager was contacted and it was learned that monensin was present in the feedmill, though not in the breeder feed formulation. Nicarbazin was not present in this feedmill. Thus, feed was collected from both the hopper on the farm, as well as a retained sample at the feedmill, to test for the presence of monensin.

Diagnostic workup and findings. Initial bacteriology samples collected during necropsy included one liver swab, three lung swabs and four bone marrow swabs. Swabs were plated on paired 5% sheep blood and MacConkey agar plates, incubated at 37°C with 7.5% carbon dioxide and no growth was reported at 24 and 48 hours of incubation. Histologic examination of trachea and lung yielded non-specific findings of tracheitis which was lymphoplasmacytic and heterophilic, and pulmonary edema and congestion respectively. Tracheal swabs collected from the initial necropsy were PCR negative for the detection of MG, MS and ILT.

Based on a clinical suspicion of calcium tetany, the veterinary team arrived for the subsequent farm visit, prepared to sample blood and test using a CG8+ cartridge on the i-STAT® Alinity V (Zoetis US, Parsippany NJ). The i-STAT Alinity V is a handheld point of care device capable of running samples deposited into specific cartridges, and will provide blood biochemical values. During the field visit, a 1 mL lithium heparin vacutainer tube (BD Microtainer, NJ, USA) and a 22-gauge needle were used to collect blood from the brachial vein of the two birds found panting and immobile. Heparinized blood was loaded into the CG8+ cartridge using a transfer pipette and then inserted into the hand-held unit. Cartridges were stored according to manufacturer stipulations. The CG8+ cartridge provides values for sodium (Na mmol/L), potassium (K mmol/L), ionized calcium (iCa mmol/L), glucose (Glu mmol/dl), hematocrit (Hct, packed cell volume [PCV]), pH, partial pressure carbon dioxide (PCO2 mmHg), partial pressure oxygen (PO2 mmHg), and bicarbonate (HCO_3^- mmol/L). Table 1 lists values obtained from a clinically normal hen and the two clinically affected hens, in the context of values published in the Manual of Poultry Diseases (1). The clinically affected hens had ionized blood calcium (iCa) values of 1.09 and 0.92 mmol/L respectively. A clinically normal hen that was sampled had an iCa of 1.52 mmol/L and a published reference range for iCa in poultry is 1.35-1.55mmol/L (1). Thus, the clinically affected birds were deemed hypocalcemic. In addition to this, while blood pH of all 3 birds sampled was within a normal range, the pCO_2 was decreased below normal and the HCO_3 was also decreased below normal levels. These findings indicated respiratory alkalosis, a physiologic compensatory change to maintain blood pH and known to contribute to the presentation of calcium tetany (2).

Tracheas collected during the farm visit necropsy were submitted for AI, NDV, and IBV-Panel PCR's at PDRC. Influenza virus and Newcastle disease virus were not detected. The PDRC IBV-Panel PCR tests for the presence of IBV (+/-) as well as the specific presence of IBV-Ark, IBV-Mass, IBV-Conn, IBV-DE072/G98, IBV-GA08, IBV-GA13 and IBV-DMV1639. The IBV (+/-) PCR was positive with a cycle threshold (CT) value of 21.1 and the IBV-DMV1639 PCR was positive with a CT of 19.8. All other PCR tests on the panel were negative.

Histologic examination of the skeletal muscle, heart, gizzard, trachea and lung yielded two major findings, which were reported the day following the PCR results; acute myonecrosis and subacute-chronic tracheitis. The myonecrosis was suggestive of a toxic injury, similar to what is observed with ionophore toxicosis, though not pathognomonic. The subacute-chronic tracheitis was nonspecific and supportive of the IBV PCR positive results. Feed testing revealed that no monensin was detected in either the sample from the farm, or the retained sample from the feedmill.

Based on the results obtained from the testing conducted it was concluded that the flock was experiencing both calcium tetany, and infection with IBV-DMV1639 concurrently. Calcium tetany explained the panting and trembling birds as well as the mortality, and IBV-DMV1639 explained the "cough" and subsequent decreased egg production with pale eggshells. It is also possible that IBV-

DMV1639 infection contributed to respiratory signs which may have exacerbated a respiratory alkalosis, thus contributing to the severity of calcium tetany in clinically affected birds.

Treatment plan. Based on the initial description of symptoms relayed to the clinical veterinary team, calcium tetany was the initial top differential diagnosis. Prior to visiting the flock, a recommendation to run additional fans while birds were feeding was made. At feeding when all hens are on the slats in a relatively small space, the ability to remove heat produced by the birds can become limited due to the density they achieve. Additionally, with feed consumption there is metabolic heat production (3), adding to the potential for heat stress, triggering respiratory alkalosis. To address suspected calcium tetany, 25-hydroxy Vitamin D3 was added to the drinking water, according to label recommendations for a duration of seven days, and feed was top dressed with oyster shell for one day. With respect to infectious bronchitis, there is no treatment for this infection and the disease must run its course through the flock. Measures of supportive care such as continued adjustment of environmental conditions to ensure bird comfort were employed.

Outcome. Mortality peaked at 0.23% in a single day, and with the addition of additional fans at feeding time, 25-hydroxy Vitamin D3 in the drinking water and top-dressed oyster shell on the feed, mortality the subsequent day was cut in half (0.12%). The mortality pattern continued to drop and cumulative mortality for the 29th week of age was 0.28%, closer to typical industry reported weekly mortality of approximately 0.25%. Overall flock peak egg production was 80.4% at 35 weeks of age, lower and later relative to standard (Figure 2). Other than pale eggs, no other egg shell abnormalities (wrinkled eggs, thin shelled eggs) were observed.

As this company was not currently employing the use of the DMV1639 custom made modified live vaccine in their broiler breeder flocks, further investigation into the potential source of viral infection was conducted. It was discovered that the week prior to the onset of clinical signs, a breeder service technician had stopped at the hatchery to collect a box of supplies. The box of supplies was located adjacent to the room where broiler chick processing was underway, and the DMV1639 custom modified live vaccine was being applied. It is suspected that despite the service person following biosecurity protocols, this box of supplies which was placed in the breeder service technicians' truck was the epidemiological link that tracked DMV 1639 to the broiler breeder flock, which was subsequently visited the next day by the technician.

ACKNOWLEDGEMENTS

The authors wish to thank the company who worked with the team at PDRC to ensure that information and samples were collected in order to arrive at a diagnosis.

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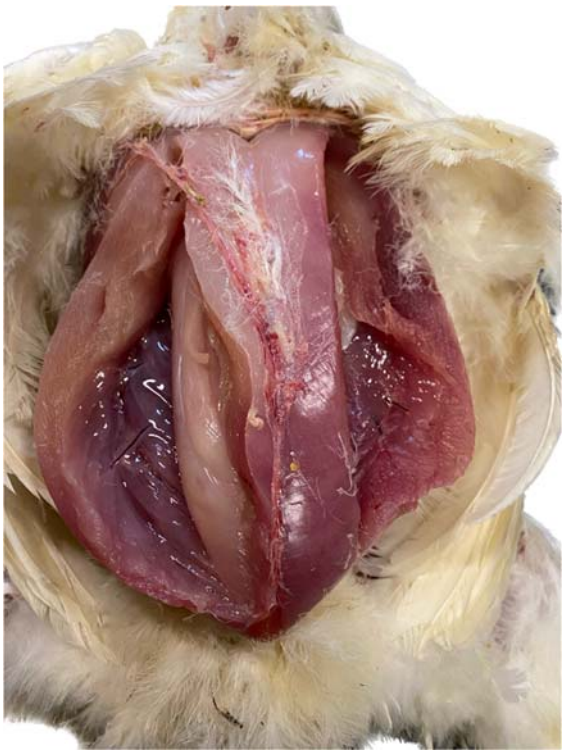
Figure 1.



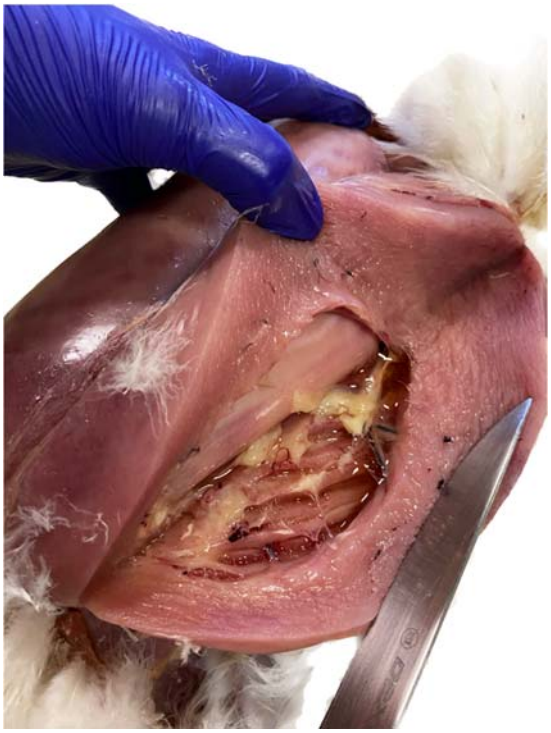
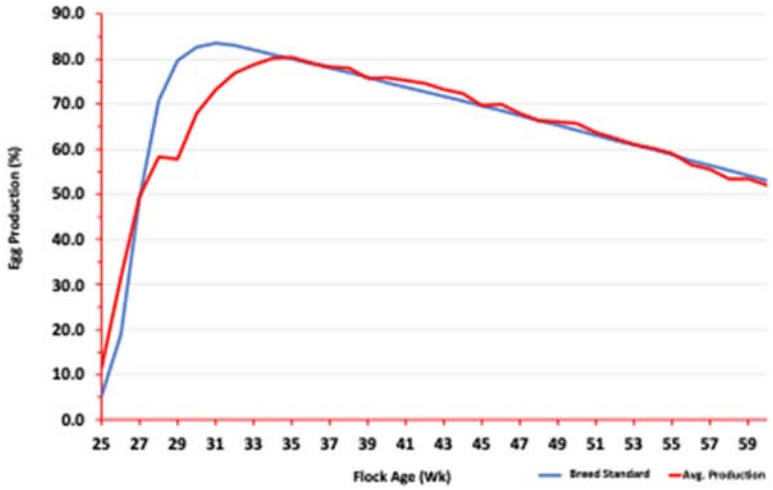


Table 1. i-Stat CG8+ values from clinically normal and affected hens, relative to available published reference values.

Parameter (units)	Reference values*	Clinically normal hen	Clinically affected hen 1	Clinically affected hen 2
pH	7.28-7.44	7.441	7.432	7.432
pCO_2 (mmHg)	40-65	37.0	23.9	25.1
pO_2 (mmHg)		56	89	62
HCO_3 (mEq/L)	24-33	25.2	15.9	16.7
BE ecf (mEq/L)		1	-8	-8
SO_2 (%)		90	97	93
TCO2 (mEq/L)		26	17	17
Na+ (mEq/L)	146-169	146	146	145
K+ (mEq/L)	4.6-6.5	4.9	4.7	5.7
iCA (mmol/L)	1.35-1.55	1.52	1.09	0.92
Glu (mg/dl)		224	255	195
Hct (%PCV)		28	32	35
Hb (g/dl)		9.5	10.9	11.9

*Manual of Poultry Diseases –Brugere-Picoux *et al*. 2015

Figure 2. Flock % egg production relative to breed standard.



INFECTIOUS BRONCHITIS VIRUS GENOTYPING IN CANADA, 2014-2022

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SUMMARY

Between 2014 and 2022 infectious bronchitis virus (IBV) partial nucleotide sequences of the IBV spike (S) protein gene were determined for 1,501 samples. Based on their S gene sequence identities, Canadian IBVs could be divided into five major groups: 1) variant viruses related to strains described in the US; (2) vaccine-like or classic viruses; 3) exotic, non-Canadian, non-US viruses, 4) "domestic" Canadian variants; 5) "unique variants" IBVs showing less than 89% identity to other IBV strains.

MATERIALS AND METHODS

Nucleic acids were extracted using the MagMAX-96 Viral RNA Isolation Kit in a MagMAX Express-96 Magnetic Particle Processor (Thermo Fisher). A real-time PCR was used as a screening test for IBV detection as described previously (1). Primers IBV_S_F-uni1_161125 (5'-GGTTGGCATYTACAHGGR-3') and IBV_S_R-uni1_161125 (5'-TCTTGTRCRGTACCATTA-3') were designed to amplify a 542 bp fragment of the IBV spike (S) protein gene from nucleotide (nt) 115 to nt 656 (based on S gene sequence from IBV 4/91, GenBank JN192154). Sequence comparison of the 507 nt fragment from nt 139 to nt 645 (based on S gene sequence from IBV 4/91, GenBank JN192154) was done using the MegAlignPro module of LaserGene software (DNAStar).

RESULTS

Genotyped samples could be classified in five major groups (Table 1): 1) variant IBVs related to strains described in the US such as DMV/1639/11, California/1734/04, CU/82792/GA98 and GA08 (n=745); (2) vaccine-like/classic viruses, such as Massachusetts, Connecticut and Arkansas (n=687); 3) exotic, non-Canadian, non-US viruses, such as strain 4/91 (n=35); 4) "domestic" Canadian variants not described elsewhere, such as strain Qu_mv (n=10); and 5) unique variants (n=24).

In commercial poultry the highest proportion of IBV strains were US variant-like (49.6%) and vaccine-like or classic viruses (47.2%). This differed from back-yard flocks where the most common findings included CAL1737-04 (31.5%) and unique (27.8%) variants. Vaccine-like viruses were detected in 7.4% of back-yard flocks. All IBVs were mutating while circulating in the field; the drift was 8.9% for 4/91, 4.0% for CAL1737-04 and 5.6% for DMV strains.

DISCUSSION

Outbreaks with 4/91, California 1734/04, and DMV/1639/11 -like IBVs were often associated with more severe disease in all chicken commodity groups (2,3). With each strain the severity of infection and number of affected flocks increased. Multiple IBV strains overlapped in their emergence, peaked and regressed, but introduction of DMV/1639/11 has resulted in a steady field challenge. It appeared that IBV DMV strain challenge reached a peak in 2019 when over 47% of genotyped samples from commercial birds were genotyped as IBV DMV. In 2020-2022 the percentage of DMV-related submissions has been steady at around 30%.

DMV strains have been continuously mutating and the challenge with DMV/1639/11 -like IBVs could not be successfully mitigated by vaccines currently available in Canada (4). Alternative vaccination protocols are being contemplated to control the field situation.

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Table 1. Summary of genotyping results of 1,501 samples from 2014 to 2022.

Group	Strain	2014	2015	2016	2017	2018	2019	2020	2021	2022
Vaccine-like/classic	Conn	1	14	7	19	49	49	37	45	20
Vaccine-like/classic	Mass	12	30	41	77	68	44	34	56	83
US variant	ARK								1	
Non-Canadian, non-US	4/91	8	5	13	8			1		
US variant	DMV		1	31	127	113	101	43	61	61
US variant	PA-Wolg98		1	3						
Canadian variant	QU-mv			5	4		1			
US variant	CAL1737-04	2	10	29	28	18	9	9	23	27
US variant	GA08							1		
US variant	CU82792			1	4	3	8	7	15	8
US variant	GA98							1		
Unique variant	BC_AHL14-023482	4	2		5				1	1
Unique variant	US_CA-K19-01179				2					
Unique variant	ON_AHL16-046445			5						
Unique variant	SH1				1					
Unique variant	Unique variant		1		2					
	Total:	27	64	135	277	251	212	133	202	200

THE MAREK'S DISEASE VIRUS (MDV) MEQ ONCOPROTEIN OF VV+MDVS SPECIFICALLY BINDS THE CHROMATIN MODIFIER BRG1 AND INCREASES MEQ TRANSCRIPTIONAL ACTIVITY

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SUMMARY

Marek's disease (MD) is a T-lymphoproliferative and immunosuppressive pathology caused by Marek's disease virus (MDV). T-lymphoma formation by MDV requires the oncoprotein, Meq, a basic leucine zipper transcription factor. The 33 amino acid C-terminal domain (CTD) of Meq contains a highly conserved transactivation domain, however, distinct mutations within an adjacent disordered proline-rich repeat region (PRR) are characteristic of virulent (v), very virulent (vv) and very virulent plus (vv+) MDV pathotypes, suggesting a role in these pathotypic differences. We hypothesized that these substitutions alter specificity or affinity of protein-protein interactions mediating a gain of function. In support of this hypothesis, we now report that the PRR of the vv+MDV Meq isoform binds a cellular ATP-dependent chromatin remodeler, BRG1, which increases transcriptional activity of the adjacent CTD. This finding provides the first experimental evidence that mutations within the PRR of the vv+MDV Meq isoform have been selected by changes in its interactome.

INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of viral etiology that progresses rapidly in domestic chicken (*Gallus gallus*). MD was clinically described as a mild neuropathology more than 100 years ago and the Marek's disease virus (MDV) agent has been in circulation for at least 1000 years (9), but concern for MD incidence and its prevention has elevated only in the last 60 years due to the severity of contemporary field strains. The industrialization of poultry production marks the incipient disease course, while continuous vaccination amounts to incessant virulence evolution. Despite a spectrum of disease severity, the main neoplastic symptoms in MD are the onset of neurological syndromes, lymphoid atrophy resulting in immune suppression, skin leukosis and development of T-cell lymphoma in visceral organs that culminate in >90% fatality within several weeks of infection in unvaccinated, susceptible chickens (22).

Concomitant with the evolution of vaccine resistant field strains requiring modifications to vaccine use, distinctive mutations were apparently selected in these breakthrough strains. The Meq oncogene is among the MDV ORFs that has evolved under positive selection (21). The oncogenic properties of Meq, an MDV-encoded basic leucine zipper (bZIP) transcription factor, have been associated with its ability to dimerize with c-Jun, inducing v-Jun responsive genes (14). The Meq C-terminus contains a proline-rich repeat region (PRR) which constitutes a highly disordered C-terminal domain (CTD). These PRRs directly affect the transcriptional function of the CTD and mediate trans-repression in and of themselves, as well as modulate the transactivation of the C-terminal 33 amino acid transactivation domain (23). The PRRs are composed of 21 amino acids flanked by proline tetrads that are analogous to SH3 or WW domain consensus binding motifs (13). The absolute number of PRRs varies according to strain of MDV encoding them, with mild and virulent strains having seven (with Meq ORFs of 398/399 aa), while very virulent (vv) and very virulent plus (vv+) having five (339 aa).

Distinct mutations within the PRR are characteristic of these MDV pathotypes. Contrary to ancestral strains, fewer proline tetrads constitute the CTD of contemporary strains by partial PRR truncation and proline to glutamine or alanine substitutions in the tetra-proline motifs at position two (P[Q/A]PP) (25). The prevailing dogma is that selection for consecutive reduction of proline tetrads affects virulence and suggests a direct role in the evolution of MDV virulence.

Functionally, polymorphisms in the PRR in addition to those in the bZIP domain serve to alter the transactivation properties of Meq (1,9,19,20,24), despite no mutations or only conserved mutations in the C-terminal transactivation domain. Moreover, in recombinant MDVs expressing different Meq

isoforms in the context of the RB-1B (a vvMDV) strain, polymorphisms in the Meq coding sequence directly affected pathogenicity and vaccine resistance (7). The mechanistic basis for how these mutations contribute to the pathotypic changes or oncogenic potential of Meq is the focus of this research. The data we present here details that mutations in the coding sequence of Meq were apparently selected to modulate the specificity or affinity of protein-protein interactions with partners involved in cell cycle regulation, chromatin remodeling, and DNA repair.

Here, we report that the central ATPase subunit of the multimeric SWI/SNF chromatin remodeling complex, BRG1, specifically binds to the Meq CTD of vv+MDVs and that this interaction confers increased transcriptional activity only in that context.

MATERIALS AND METHODS

Plasmid construction. To generate the constructs expressing chicken HA-tagged BRG1 (SMARCA4), the SMARCA4 CDS was amplified by PCR as two partial fragments, with 50 bp of homology at the 3' ends of each fragment, from cDNA templates isolated from MDV-infected chicken specimens (Md5-infected CEFs) using Platinum SuperFi II DNA polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA) under the conditions recommended by the manufacture. Primer pairs (forward/reverse) are as follows: (5'-GCTAGC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT ATGT CGA CCC CGG ACC CCC C-3'/ 5'-CGA ACT CGT AGG CCC AGT TGG AGA GAG TTG AG-3') and (5'- CCA GAC CAT CGC GCT CAT CAC GTA CCT CA-3'/ 5'-GAATTC TCA AAGCTT GTC CTC CTC CGT GCC GCT GCC GGA GCG CTC-3'). Primers used for mutagenesis were synthesized corresponding to the 5'- and 3'-ends of SMARCA4, to which unique restriction sites (underlined) were included upstream of the start codon (NheI) and flanking the stop codon (HindIII/EcoRI). The forward primer appends the HA sequence to the N-terminus while the reverse allows for the discretionary generation of C-terminal fusion constructs of BRG1. The full-length SMARCA4 CDS was assembled by overlapping PCR using the outermost primers (bold face), followed by cloning the entire coding region into pCR2.1 TOPO vector (Invitrogen). Expression vectors for HA-tagged SMARCA4 were constructed by restriction digestion and ligation into pBK-CMV vector (Stratagene) via NheI and EcoRI sites; or into the pECFP-N1 (CLONTECH) vector via NheI and HindIII sites to generate an N-terminal fusion to ECFP. The construction of all SMARCA4 plasmids were validated by DNA sequencing. The T7-tagged Meq expression plasmids (from MDV strains JM102, 617A, RB-1B, TRPLΔ, and N Meq isoforms) were previously generated by subcloning into pBK-CMV vector for the full-length Meq CDS (11) and pEYFP-N1 vector for Meq deletion mutants (RB-1B Meq, Meq/vIL8, Meq/vIL8Δexon3, Meq bZIP, and Meq/vIL8 bZIP) (2).

For luciferase assays, fusion expression constructs were generated by cloning the Meq C-terminal transactivation domain, corresponding to amino acids 121-339 (RB-1B and N Meq) or 121-398 (JM102 Meq), in frame with the GAL4 DNA binding domain (GAL4-DBD) into pSG424 vector. For generating the experimental reporter plasmid for luciferase assays, the Red Firefly luciferase cassette was digested from pMCS-Red Firefly luciferase (Pierce) and inserted downstream of the E1B minimal promoter containing 5X Gal4 upstream activation sequence. The TK promoter-driven Renilla luciferase reporter (pTK-Green Renilla, Pierce) was used as an internal control.

Cell lines, cell culture conditions, and transfections. CU91, MSB-1, UD35, and UA53 lymphoblastoid cell lines were maintained in Iscove's modified Dulbecco medium (IMDMEM) supplemented with 20% fetal bovine serum (FBS; R&D Systems, Inc, Minneapolis, MN), 1X insulin-transferrin-selenium, 1X nonessential amino acids, 2 μM β-mercaptoethanol, 4 mM L-glutamine, 0.5 μg/mL antimycotic (amphotericin B, Corning, Corning, NY), and 1X Penicillin, Streptomycin, Neomycin (PSN) antibiotics at 41°C with 5% CO2 in a humidity controlled incubator.

For transfection and fluorescent protein visualization experiments, the chicken macrophage cell line HD11 was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (R&D Systems), 0.5 μg/mL amphotericin B, and 1X PSN (except where stated, reagents were manufactured by Gibco, Thermo Fisher Scientific, Waltham, MA) at 37°C with 5% CO2.

HD11 cells were seeded into 12-well plates at a density of 2 x 105 cells/well for colocalization experiments or 96-well black tissue culture assay plates at a density of 1 x 104 cells/well for dual-luciferase reporter assays and transiently transfected once cells reached 65-75% confluency using 1 μg Lipofectamine 2000 Transfection Reagent (Invitrogen). The DNA-liposome complexes were prepared in serum- and antibiotic-free DMEM according to manufacturer's suggestions. Cells were incubated to 4 hrs post transfection at 37°C prior to the addition of fresh DMEM complete growth media and allowed to grow for 24 hrs.

Immunoprecipitation and proteomics. For endogenous immunoprecipitation studies, whole cell

lysates were prepared from MD lymphoblastoid cell lines CU91, MSB-1, UD35, and UA53. Cells were harvested, washed with ice cold 1X PBS then lysed in RIPA buffer supplemented with DTT, protease and phosphatase inhibitors by performing three rapid freeze thaws. Lysates were incubated with 40 µg of DNase I for 1 hr at 4°C prior to clearing the lysate. From lysate supernatants, candidate Meq binding partners were immunoprecipitated with 50 µL of rabbit polyclonal anti-Meq serum in RIPA wash buffer overnight at 4°C with constant inversion and followed by inverting with a 50% slurry of protein A/G agarose resin (Pierce) for 2 hrs at 4°C. The precipitants were then washed three times with RIPA wash buffer, then pelleted and snap frozen in LN2 prior to shipment for liquid chromatography-tandem mass spectrophotometry (LC-MS-MS)-based proteomic analysis (MZ-Biolabs). Peptides were mapped to the chicken, MDV, HVT, and SB1 genomes and anti-Meq interactomes were filtered against the CU91 proteome to eliminate non-specific peptides.

Bioinformatic analysis of proteomes. For gene ontology enrichment analysis, accession numbers for identified proteins were annotated for terms according to category of biological process (BP), molecular function (MF), and cellular component (CC) using the Gene Ontology database.

Immunofluorescence analysis. HD11 cells were co-transfected with equal molar amounts of expression plasmids for pBK-HA-SMARCA4 along with the pBK-CMV empty vector or the following Meq isoforms; pBK-T7-JM102 Meq, pBK-T7-617A Meq, pBK-T7-RB-1B Meq, pBK-T7-TRLPΔ Meq, and pBK-T7-N Meq. Cells were allowed to grow for 24 hrs to fill in prior to fixation in 1% paraformaldehyde. Fixed cells were washed three times in 1X PBS, then blocked in 1X PBS, pH 7.4, 3% goat serum, 1% BSA, 0.1% NaN3 with 0.1% saponin to permeabilize the membrane for 2 hrs at room temperature with gentle rocking. Cells were stained with primary antibodies diluted in blocking buffer for 1 hr at room temperature (1:100 rabbit anti-Meq polyclonal serum and 1:100 mouse anti-HA tag). Meq antisera were generously provided by Dr. Hans Cheng (USDA-ADOL) and were pre-adsorbed sequentially against ethanol-fixed CU91, CEF, HD11 and HTC cell lines. Cells were washed three times with 1X PBS, pH 7.4, 1% BSA, 0.1% NaN3 prior to staining with secondary antibodies (1:200 Goat anti-rabbit conjugated with Alexa 555 and Goat anti-mouse Alexa 488; Molecular Probes, Thermo Fisher Scientific, Waltham, MA) for 1 hr at room temperature. Following antibody staining, cells were counterstained with 1X PBS, pH 7.4, 10% glycerol, and 6 nM DAPI.

For domain mapping localization experiments, Meq splice variants and domain mutants were expressed as an N-terminal fusion to EYFP. Equal amounts of expression plasmids (100 ng) for pEYFP-N1 empty vector, Meq, Meq/vIL8, Meq/vIL8Δexon 3, Meq bZIP, Meq/vIL8 bZIP were co-transfected along with pHA-BRG1-ECFP. Transfected cells were grown for 24 hrs post transfection prior to fixation in 1% paraformaldehyde. Fixed cells were washed three times with 1X PBS then counterstained with 1X PBS, pH 7.4, 10% glycerol and 6 nM DAPI prior to imaging. Image acquisition was performed with a Nikon Eclipse TE2000-U inverted epifluorescence microscope with a Plan Fluor 20X objective and Nikon NIS Elements imaging software (v5.02).

Transcriptional Activation Analysis HD11s were co-transfected with pBK-CMV empty vector or BRG1 (50 ng) along with the dual reporter Red Firefly luciferase/Green Renilla luciferase plasmids (50 ng/10 ng) and the GAL4-DBD alone (pSG424 empty vector, Stratagene) or as an N-terminal fusion to the Meq CTD of the JM102, RB-1B, and N strain Meq isoforms (100 ng). The total amount of transfected plasmid DNA (300 ng) was kept constant using the pUC19 vector. Luciferase reporter activity was measured using the Renilla-Firefly Luciferase Dual Assay kit (Pierce) according to the following modifications. Twenty-four hours post transfection, medium was aspirated, and monolayers were washed with ice cold 1X PBS prior to lysis in 25 L of 1X cell Lysis Buffer for 15 minutes at room temperature with rocking. The Luciferase Dual assay was conducted in a SpectraMax i3x plate reader (Molecular Devices), programmed to inject 25 L of Working Solution A containing 1X D-Luciferin and to detect luminescence to 650 nm, then samples were read again with the monochromator tuned to 535 +/- 15 BP immediately following the manual dispensing of 25 L of Working Solution B containing 2X Coelenterazine. Relative light units for Firefly luciferase were normalized to Renilla luciferase and fold activation is relative to the pGal4-DBD empty vector. Luciferase assays were performed in triplicate with each experiment repeated three times.

RESULTS

Meq interactome. To profile the interactomes of Meq isoforms we conducted MS-based proteomic analysis on anti-Meq co-immunoprecipitants of MD lymphoblastoid cell lines derived from lymphomas of chickens infected with BC-1, RB-1B, and TK MDV strains (Figure 1). Notably, we found that the v, vv, and vv+MDV Meq interactomes differ appreciably, suggesting that the oncogenic potential of Meq is affected by discrete interaction networks mediated by specific mutations.

With respect to the transforming agent, the strain pathotype, and the Meq isoforms expressed by these

cell lines, functional gene ontology analysis revealed that host cellular factors involved in chromatin remodeling and DNA repair mechanisms were enriched biological processes targeted by vv+MDV Meq isoforms.

These findings prompted our investigation of key chromatin remodeling and co-activators with known disease associations; namely, SWI/SNF complexes. Of these complexes, the BRG1 subunit was overrepresented in the proteome of CD30HI MDV transformed T-reg-like lymphoma cells (5).

To complement our proteomic analysis, we cloned candidate interactors and subsequently validated their interaction dynamics with Meq isoforms by performing bioimaging studies.

Localization, colocalization, and domain mapping. To characterize the localization properties of the chicken BRG1 (Figure 2), we expressed BRG1 as an N-terminal fusion to EYFP in HD11 cells and performed localization analysis. Like its mammalian homolog, the localization of the chicken BRG1 is also restricted to the nucleoplasm and excluded from subnuclear domains.

Given the cytoplasmic to nuclear, and subnuclear translocation properties of Meq (2,16), we investigated the colocalization dynamics of BRG1 in the presence of v, vv, and vv+MDV Meq isoforms, containing unique CTDs (Figure 2A). In reciprocally tagged colocalization assays, we evaluated the putative association of EYFP- or ECFP-tagged BRG1 and Meq isoforms in the nucleus and nucleolus of HD11 cells.

In stark contrast to BRG1 alone, the Meq isoforms indiscriminately caused the relocalization of BRG1 to the nucleolus. Although, the presences of Meq splice variants (Meq/vIL8 and Meq/vIL8Δ exon 3) and Meq domain mutants (Meq bZIP, Meq/vIL8 bZIP), all lacking the CTD, resulted in no such nucleolar localization of BRG1. These colocalization studies provide evidence that implicated the C-terminal 219 aa in targeting BRG1 to subnuclear domains, and that the Meq bZIP domain alone or with exons 2 and 3 of vIL8 are not required for this function (Figure 2B).

The C-terminally EYFP-tagged Meq isoforms can contribute to non-specific interactions with BRG1, thus we reasoned that the fluorescent protein tags are possibly obscuring our interpretation of BRG1 specific binding sites within the unique CTDs by overcoming the subtle differences in binding affinities among Meq isoforms. To map the residues in the CTD responsible for BRG1 mislocalization, we conducted colocalization experiments by expressing the epitope-tagged versions. Nuclear and subnuclear colocalization was examined by immunofluorescence analysis of BRG1 with Meq isoforms encoded by the vMDV (JM102), vvMDV (RB-1B), and vv+MDV (N) strains.

Interestingly, the N strain Meq specifically colocalized with BRG1 in the nucleoplasm and nucleolus. To determine specific Meq CTD mutations mediated BRG1 localization, we employed the 617A Meq and the RB/N chimeric Meq (TRPLΔ). The former encodes a P217A substitution in a proline tetrad that is proximal to the transactivation domain. The latter differs from RB-1B Meq at positions corresponding to the first two proline tetrads, P153Q and P176A, in addition to a P217A substitution, which are the three CTD residues conserved by vv+MDVs.

Like the vMDV and vvMDV Meq isoforms, the 617A Meq and BRG1 were differentially localized in the nucleolus, suggesting that the C119R substitution a Retinoblastoma (Rb) binding pocket in addition to the P217A substitutions are dispensable for BRG1 nucleolar translocation. Remarkably, BRG1 and the TRPLΔ Meq mutant, with the second site substitutions introduced in three of the five proline tetrads of the RB-1B Meq isoform, exhibited colocalization properties consistent with vv+MDV Meq isoform in the nucleolus. Together, these domain and mutagenesis data indicate that residues at positions 153, 176, and to a lesser degree 217 in the Meq CTD mediate BRG1 interactions, independently of the Rb binding pocket or bZIP domain, causing its localization to the nucleolus (Figure 2).

Transactivation assay. To investigate the functional relationship between BRG1 and the CTD of Meq, we assayed the transactivation potential of Meq CTDs encoded by JM102, RB-1B, and N strains alone or in the presences of BRG1 in GAL4 DBD-based *in vitro* transactivation assays. The CTD of v, vv, and vv+MDV Meq isoforms induced modest, but not significant, incremental increases in transactivation potential that corresponded with virulence level. The coexpression of BRG1 significantly increased the transactivation potential of the vv+MDV Meq CTD by a ~12-fold increase (data not shown), whereas v and vvMDV Meq CTDs were unaffected. These results indicate a role for BRG1 coactivation and implicate the PRR, specific second-site mutations as a critical component to the properties of the increased Meq transactivation activity associated with the vv+ isoform.

DISCUSSION

Our work presents the first comprehensive study of the Meq interactome, with a focus on proteins having increased affinity for Meq proteins having point mutations in the proline tetrads of the PRR.Our work describes a functional mechanism by which the PRR participates at the interface of intermolecular

interactions in concert with cellular coactivators of the SWI/SNF chromatin remodeling complex.

Oncogenic serotypes of MDV share significant homology in the Meq coding sequence. Albeit, v, vv, and vv+MDV pathotypes are phylogenetically divergent due to polymorphisms at conserved positions in the CTD of Meq. The CTD comprises two moieties, the PRR and the transactivation domain, offering pleiotropic properties that regulate target gene repression and activation, respectively (23). These properties are not mutually exclusive in that the transactivation domain demonstrates the requirement of at least partial PRR cooperation in transcriptional regulation. Paradoxically, the isoforms from our analysis demonstrate considerable differences in transactivation potential despite conservation within the integral transactivation domain, thereby prompting our investigation on the adjacent unique PRRs.

Previous studies implicated the contribution of the tetra-proline motifs and their cognate targets to transcriptional regulation. This ambiguous role for the proline tetrads, in turn, precludes our understanding of a mechanistic basis for driving the oncogenic potential of Meq. Here, we identify previously unknown cellular interactors of the Meq interactome and validate them by orthogonal approaches. Our results imply that the introduction of alternative residues at positions 153, 176, and 217, disrupting three proline tetrads, mediates the specificity for binding to BRG1. The perturbation of BRG1 localization by Meq-mediated translocation serves as a proxy for binding affinity among isoforms while also indicating an alternative function for BRG1 in the nucleolus. BRG1 has been implicated in the repression of active sites of transcription in response to DNA double stranded breaks (DSB) (10). Molecular sequestration of BRG1 in the nucleolus would likely affect this function in response to DNA damage. Additionally, our experiments demonstrate that the vv+MDV Meq protein recruits BRG1 and together exhibit increased transactivation activity, which clearly demonstrates a mechanistic role for partial PRR segments in transactivation. We find this preference for BRG1 to be highly suggestive of a cooperative mode of action between these transcription factors via specific residues in the PRR of Meq and thereby a mechanism by which the vv+MDVs have emerged.

Interestingly, by using MobiDB-lite to score disordered residues, the Meq CTD has a predicted intrinsically disordered region (IDR) spanning residues from positions 145 to 169. Thus, we speculate that the local structure of the PRR is affected by the P153Q substitution that corresponds to a proline tetrad embedded within the IDR. Our prediction analysis suggests a shift in disorder further reducing the conformational order within this region relative to a proline residue at this position of RB-1B and 617A strains. The current perspective is that selection pressures for Meq follow a structure-function relationship whereby substitutions P176A and to a lesser extent P217A increase order. We argue, however, that the evolutionary biases for position 153 follows the disorder-function model. Taken together, the P/Q substitution permits an altered conformational state of the IDR directly affecting its protein-protein interactions.

Previous studies by our group and others, demonstrate that mutations in Meq gene of recombinant MDVs are sufficient to confer pathotypic alterations (6,7). Furthermore, the mechanisms coordinating MD tumor formation are not only regulated by Meq (18), but also through properties of its protein interaction network (3,4,8,12,15,17,26–28). The distinct interactomes of Meq isoforms reported in our study provides functional insight into the divergent paths of MDV evolution, through which preferred mechanisms are leveraged for oncogenesis. For instance, the substitutions in the CTD of vv+MDV Meq may counter the consequential loss for RB binding due to the C119R substitution in the RB binding pocket (LxCxE). While the significance for this role is not yet fully understood, it is tempting to speculate that recruitment of BRG1 by vv+MDV Meq isoforms could - by a gain-of-function mechanism - endow transactivation properties to Meq homodimers at MEREII sites to regulate cellular malignant reprogramming or viral dynamics.

In summary, our study provides the first mechanistic basis for how mutations in the PRR common to the CTD of Meq proteins encoded by vv+MDVs mediate increased transcriptional activation. This is the first evidence of a virus-encoded bZIP protein mediating changes in transcriptional activation via this mechanism.

ACKNOWLEDGMENTS

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Figure 1. Summary of differentially identified proteins from anti-Meq interactomes of lymphoblastoid cell lines derived from v, vv, vv+MDV-induced lymphomas. Shown is the comparative MS-based proteomic analysis of the anti-Meq co-immunoprecipitants from MSB-1, UD35, and UA53 cell lines, representing the endogenous Meq binding profiles of isoforms encoded by BC-1, RB-1B and TK MDVs. The resulting interactomes were evaluated by GO enrichment analysis.

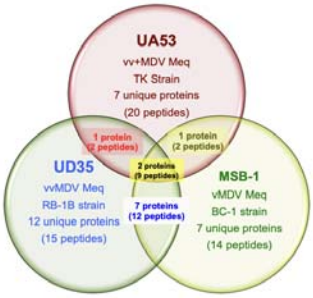
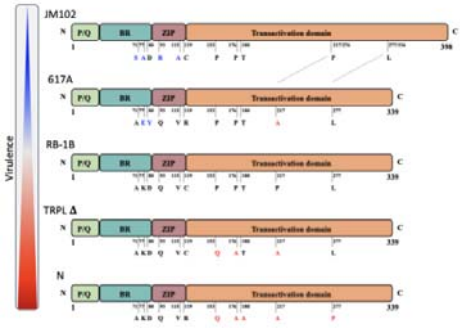


Figure 2. Localization dynamics of BRG1 with Meq Isoforms. (A) Meq isoforms and their respective CTD substitutions used in colocalization studies. (B) Colocalization summary of Meq isoforms and domain mutations with the chicken BRG1(SMARCA4); N, nucleus; C, cytoplasm.

A



B

		+ SMARCA4	
Meq isoform	Expression construct	Subcellular localization	Nucleolar localization
Vector	pBKCMV	N>C	-
	pECFP/EYFP	N>C	-
JM102	pBKCMV	N>C	-
	pECFP/EYFP	N>>C	+
617A	pBKCMV	N>C	-
	pECFP/EYFP	nd	nd
RB1B	pBKCMV	N>C	-
	pECFP/EYFP	N>>C	+
TRPLΔ	pBKCMV	N>C	+
	pECFP/EYFP	N>>C	+
N	pBKCMV	N>>C	+
	pECFP/EYFP	N>>C	+
Meq/vIL8	pBKCMV	nd	nd
	pECFP/EYFP	N>C	-
Meq/vIL8ΔExon 3	pBKCMV	nd	nd
	pECFP/EYFP	N>C	-
Meq bZIP	pBKCMV	nd	nd
	pECFP/EYFP	N>C	-
Meq/vIL8 bZIP	pBKCMV	nd	nd
	pECFP/EYFP	N>C	-

RAPID POULTRY DISEASE DIAGNOSIS USING METABOLOMICS

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SUMMARY

Infectious diseases cause significant economic losses to the Canadian poultry industry. The inability to diagnose diseases at early onset remains a challenge and leads to economic losses and welfare issues. Metabolic biomarkers can be used as an early warning system considering pathogen-induced metabolic changes. In human medicine, metabolomic studies are common while in veterinary medicine they are still in their infancy. The objective of this study was to identify the metabolomic landscape of broiler chickens following infection with *Escherichia coli* and avian reoviruses. We analyzed the serum metabolomic profile of chickens at 24 hours following infection with *E. coli* or avian reovirus using liquid chromatography-mass spectrometry. We observed a clear separation of metabolites between infected and uninfected birds particularly the downregulation of the purine metabolism pathway. This is likely associated with energy metabolism and signaling during cellular stress. These metabolites are promising biomarkers for early detection of *E. coli* and reoviral infection in broiler chickens and assist in implementing early interventions to minimize economic losses.

INTRODUCTION

The chicken industry has been using small preventative doses of various antibiotics as feed additives to prevent diseases. However, when the prophylactic use of antimicrobials is stopped, the infection rate of pathogens like *Escherichia coli* and *Clostridium perfringens* increases adversely affecting the chicken's health and welfare (1). One of the major challenges in the Canadian poultry industry is the lack of a powerful modality for the early detection of subclinical infections before disease outbreaks. The chicken industry mostly relies on serological blood testing to measure antibodies to detect pathogenic infections. However, serological tests detect diseases only 10-14 days after pathogenic exposure. Besides,

PCR (pathogen's DNA detection) and bacterial culture-based diagnosis methods are primarily contingent on the types of tissue and the pathogen's predilection site. The Canadian chicken industry cannot detect pathogens within 1-2 days post-infection. Viruses lack an independent metabolism and must instead utilize host resources to support their proliferation which leads to changes in host metabolites. Bacteria have an independent metabolism, and they produce bacterial-specific metabolites. However, the novel techniques of metabolomics opened new avenues in disease diagnosis by allowing early detection of human and animal diseases using metabolic biomarkers. Thus, validated measurable metabolic biomarkers can interpret disease risk, early onset, progression, and prognosis (2). Although the previous investigations mostly focused on chickens' metabolic syndromes (3-5), the metabolomics landscape associated with viral, and bacterial infectious diseases is limited. Avian colibacillosis and viral arthritis are two common infectious diseases in the broiler industry leading to loss of production. Therefore, this study aimed to identify broiler chickens' metabolomic landscape and potential biomarkers for early detection during infection with *E. coli* or avian reoviruses.

MATERIALS AND METHODS

All animal experiments were conducted following approval by the Animal Research Ethics Board of the University of Saskatchewan. The study was conducted using broiler chickens (Ross 308). All the challenge experiments were carried out in the level two facility of the Animal Care Unit, University of Saskatchewan. Day-old birds obtained from a commercial hatchery were maintained under controlled environmental conditions with strict sanitation per Aviagen guidelines.

***E. coli* challenge experiment.** As previously described by Gunawardhana *et al*. (6), avian pathogenic *E. coli* of serotype O2, isolated from a field case of turkey was used for the challenge. Briefly, bacteria were streaked on blood agar and incubated aerobically at 37^0 C for 24 hr. Then, one or two colonies from blood agar were transferred to 100 mL of Luria broth (Difco LB broth, Miller, Becton Dickinson, and Company; USA) and incubated aerobically at 37^0 C in a shaker with 150 rpm for 16-18 hr. until the stationary phase 10^9 colony forming units (CFU)/mL was obtained. The bacterial broth containing 10^9 CFU/mL was diluted in phosphate-buffered saline until the challenge dose of 10^5 CFU/mL was obtained.

Birds (n=30) were challenged on day five post-hatch by administering 10^5 CFU/mL of the prepared *E. coli* in 250 µL per bird subcutaneously. The negative control group (n=30) was kept uninfected. Birds were observed for the development of the clinical signs post-challenge. Sampling was done at 24 hours post-challenge.

Reovirus challenge experiment. In a separate experiment, the avian reovirus used for the challenge was a cell culture-grown field isolate from Saskatchewan. A total of 10^5 plaque-forming units (PFU)/mL of reovirus in 100 µL per bird (n=30/ time point) was administered into the right foot pad as previously described by Ayalew *et al*., (7). The negative control birds were kept uninfected (n=30/time point). The birds were observed for lameness, foot pad, and joint lesions following the challenge. Sampling was done at two time points 24 hr and 72 hr post-infection.

Sampling. Blood was collected from the birds in serum tubes and euthanized by cervical dislocation. Post-mortem was performed on the birds to observe any gross lesions. Individual blood samples were centrifuged at 1000 rpm for 10 min to separate the serum. The serum was flash-frozen in dry ice immediately and stored at -80^0 C until the metabolomics analysis was performed.

Metabolomic analysis. The deproteinized serum samples were run on a liquid Chromatography mass spectrometer for untargeted metabolomics. Data analysis was carried out using Metaboanalyst 4 software using multiple statistical approaches including univariate, multivariate [principal component analysis (PCA)], partial least square discriminant analysis (PLS-DA), feature selection, and machine learning methods to detect significant metabolite alterations in the treatment group in comparison to the control.

RESULTS

No clinical signs were observed in birds 24 hr post-challenge in both *E. coli* and reovirus challenge experiments. However, at 72 hrs, the reovirus-challenged birds showed moderate-severe foot pad lesions and lameness. The remaining 3.3% showed mild-moderate foot pad lesions.

PCA and PLS-DA analyses were used to compare the metabolomic differences between the "control" and "infected" groups. They revealed a good class separation with minimal overlap based on the components chosen. Variable importance in projection (VIP) scores greater than one was considered highly significant and led to the differentiation of metabolite classes. PCA and PLS-DA's data dimensionality was further decreased by feature selection and machine learning approaches. Finally, metabolite network and pathway analysis revealed that the purine metabolism was downregulated in both *E. coli* and reovirus-infected chickens.

DISCUSSION

The metabolome is sensitive to rapid changes in the body and detects metabolite changes at the cellular level as a pathogen enters the body, thereby reflecting an individual's metabolic status and physiological activities during early infection (2). Further, Immunometabolism is a related study area that interprets the association between metabolism and immune mechanisms of organisms. During the early stages of infection, there will be a reprogramming of energy generation and biosynthesis, including an increase in glycolysis to ensure faster ATP production and biosynthesis for damage repair and defensive response. Additionally, epigenetic modifications connected to immune cell proliferation, signaling, activation, and differentiation will take place. At the same time, high reactive oxygen species concentrations during the early inflammatory phase cause damage to cells and DNA which leads to the need for DNA repair (8). Purine metabolism plays a significant role in energy generation, DNA synthesis, and signaling in the body. Therefore, the purine metabolism is highly likely to alter during the early phases of infection. Hence it can be used as a potential biomarker for early detection of colibacillosis and reovirus infection in chickens.

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AVIAN METAPNEUMOVIRUSES REVISITED: CHALLENGES IN DIAGNOSIS AND CONTROL

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ABSTRACT

Based on nucleotide and deduced amino acid sequence analyses avian metapneumovirus (AMPV) is assigned to the genus *Metapneumovirus* together with the human Metapneumovirus (HMPV), which was suggest to have evolved from virus of bird origin. Different subtypes (A-D) have been described in poultry in various regions in the world. Different poultry as well as wild bird species show variable susceptibility to the different subtypes. New subtypes were suggested recently for viruses detected in gulls and parakeets. AMPV shows a strong tropism for epithelial cells in the trachea and turbinates as well as in the reproductive tract, which leads to respiratory symptoms and reproductive disorders, and subsequently significant economic losses. The clinical signs are often mild. They affect only part of the flock but can be exacerbated by secondary bacterial pathogens, which is then the main trigger for veterinary investigations. AMPV infections may be difficult to diagnose in the field due to an only short detection period in choanal and tracheal swabs depending on the virus strain and host. In addition, the virus seems to be fairly unstable in diagnostic material, which affects the detection rate and the chance for virus isolation explaining the low number of available laboratory and AMPV vaccine strains. Today various in-house procedures but also commercial (q) RT-PCR systems are available to detect the different subtypes in multiplex assays, but it has to be considered that newly emerging subtypes may be missed depending on the design of the RT-PCR reaction.

While subtype D was detected only retrospectively in samples from the 1980th, subtype A and B still circulate in many regions in the world since their first description at the end of the 1970th. Different subtype C strains emerged in the US in the middle of the 1990th and later in Asia and Europe, the latter continuing to spread further. Depending on the bird's species and density of the poultry population in a specific region control strategies may vary. In poultry dense regions, biosecurity seems to be less efficient for the control of the virus and therefore, vaccination is additionally implemented. In regions with less poultry flocks and low field pressure, eradiation strategies and biosecurity may control AMPV spread. It seems to be important to also understand the circulation of the viruses in wild bird populations, especially the ones having their habitat in or near farming regions, as they may support virus presence. So far, only attenuated live vaccines and inactivated vaccines are commercially available to protect against different subtype, the latter especially for use in laying birds to prevent losses in egg production. The relatively short duration of local immunity after the application of live vaccines may require frequent booster vaccinations especially in long-living birds. Cross-protection has been observed between subtype A and B. It has to be considered that vaccine usage may drive virus evolution in the field, and even reversion of vaccine strains to more virulence was observed. Experimentally, new generation vaccines were tested. The development of reverse genetics systems for AMPV of different subtypes may allow the design of new candidate vaccines in the future with reduced safety concerns and long lasting protection compared to the currently available ones.

DELIVERY OF PROBIOTICS AS A COARSE SPRAY ON INCUBATING CHICKEN EGGS AS A POTENTIAL TECHNIQUE TO IMPROVE COLONIZATION OF PROBIOTICS IN CHICKEN EMBRYOS

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SUMMARY

With the phasing out of antimicrobial use, alternatives to antimicrobials and innovative technologies are needed to control bacterial diseases in the chicken industry. The objective of this study was to explore the possibility of delivering probiotics to chicken embryos as a noninvasive technique to promote intestinal health against infectious diseases in neonatal chickens. This study was conducted by spraying probiotics (*Enterococcus faecalis, Bifidobacterium gallinarum, Pediococcus acidilactici*, and *Lactobacillus salivarius*) on specific pathogen free (SPF) eggs on the 12th, 15th, and 17th day of embryonation (DOE) at the concentration of $1x10^9$ colony forming units (CFU)/ml (log phase bacteria) spray solution for 30 seconds. Matrix assisted laser desorption-time of flight (MALDI-TOF) and whole genome sequencing was used to confirm the colonization of probiotics in the intestine of chicken embryos. Spraying probiotics twice on incubating eggs appear to be effective on colonization in the intestines of embryos. We have demonstrated that spraying probiotics on incubating chicken eggs as a feasible technique to promote colonization of probiotics.

INTRODUCTION

The preventive use of antibiotics has been discontinued in Canada; however, therapeutic use is still required to treat many infectious diseases in poultry. The Canadian broiler chicken industry is transitioning into antimicrobial free farming (1). It has been found that *Enterococcus faecalis* was able to penetrate through the egg shell (2). In this experiment, embryonated eggs were dipped in *E. faecalis* broth (temperature 10^0C) for 30 seconds on Day 12. Based on this model, bacteria can be introduced to the embryo by application on the eggshell. This study proposes a novel approach of introducing probiotic bacteria during incubation as a coarse spray application on hatching eggs to minimize pathogenic bacterial colonization in the intestinal tract of newly hatched chicks. This study will find whether probiotic bacteria can migrate to intestine of embryo when applied as a "coarse spray."

MATERIALS AND METHODS

E. faecalis, Bifidobacterium gallinarum, Pediococcus acidilactici and Lactobacillus salivarius were delivered to embryonating specified pathogen free (SPF) eggs. Bacterial broths were maintained at 10^OC and were sprayed over eggshell for 30 seconds on 12, 15 and 17 days of embryonation (DOE).

The DOE for optimal spraying, the interval between two sprays and number of repeated times of sprays were determined to investigate the DOE and number of repeated sprays need for the colonization of probiotic bacteria in the intestine of chicken embryos. Accordingly, one time spray was conducted either 12, 15 or 17 DOE. Two times spray was conducted either 12 and 15 DOE or 15 and 17 DOE. Spraying was repeated on 12, 15 and 17 DOE for three days spray group. As a positive control, eggs were dipped in an E. *faecalis* broth for 30s as previously described (2). The negative control group was not sprayed. All groups of eggs were contained in separate incubators. The migration of probiotic bacteria through the eggshell into intestine was measured by culturing intestine of embryos at 20 DOE using selective media and identification by matrix assisted laser desorption-time of flight (MALDI-TOF) and whole genome sequencing.

RESULTS

E. faecalis was able to colonize the intestine of chicken embryos by 20 DOE after spraying on the eggshell at two repeated days and three repeated days during the incubation period. A total of 90% of the embryos were positive for *E. faecalis* on selective

media after spraying with *E. faecalis* cultures when sprayed 3 repeated days (12, 15 and 17 DOE), 94.4% embryos positive for *E faecalis* on selective media after spraying with *E. faecalis* on 12 and 15 DOE, 75% embryos positive for *E. faecalis* on selective media after spray with *E. faecalis* at 15 and 17 DOE, and 60% of embryos were positive for *E. faecalis* on selective media in the positive control group. None of the other groups were able to give direct cultures of respective bacteria in selective media. The negative control group was not given any direct cultures on blood agar. Whole genome sequencing of sprayed bacteria and direct cultured bacteria proved the colonization of probiotic bacteria in the intestine of chicken embryos when probiotics were sprayed on the eggshell during incubation period with an average of 100% nucleotide identity.

DISCUSSION AND CONCLUSION

Delivery of probiotic bacteria as a coarse spray on incubating eggs is a promising technique for the colonization of probiotic bacteria in the intestines of chicken embryos. Further studies need to be conducted to find the effect for the microbiome and the ability of colonized probiotic bacteria to act against pathogenic bacteria.

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SURVIVABILITY OF HIGHLY PATHOGENIC AVIAN INFLUENZA, H5N1, IN TURKEY CARCASSES FOLLOWING DEPOPULATION AND COMPOSTING

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ABSTRACT

Highly pathogenic avian influenza continues to be a challenge for poultry producers in the United States. This present study looks at virus persistence and viability within turkey carcasses following depopulation on an infected premises. Current data supports that virus particles remain present within the trachea and cloaca for up to six days, however virus isolation data is in progress and the results will provide additional information regarding virus viability following euthanasia and composting.

INTRODUCTION

Highly pathogenic avian influenza continues to be a top concern for poultry producers across the United States and Canada. During this current outbreak with H5N1, the majority of cases have occurred through the introduction of the virus via wild migratory birds (1). From January 2022 to December 2023, USDA APHIS reported over 8000 wild bird detections and over 1000 commercial and backyard poultry cases with nearly 78 million birds being affected (2). Biocontainment of the virus is vital in reducing the spread of disease during an outbreak from farm to farm. A previous field study found that virus viability in turkey carcasses following euthanasia lasted for three days. Afterwards virus isolation was not possible even with particle detection through RT-PCR (3). To increase our understanding of the viability of the virus and improve control in the event of an outbreak, this study looked at the presence and viability of HPAI in clinically and non-clinically affected commercial turkeys with and without composting during an outbreak in October of 2023.

MATERIALS AND METHODS

During depopulation of the affected house, 15 most clinically affected birds were painted black ("B", 8 of those died prior to CO2), 12 clinically affected birds were painted green ("G"), and 24 less or non-clinical birds were painted orange ("OR") for a total of 51 birds enrolled in the project. Individual tracheal and cloacal swabs were taken and all birds alive after selection were depopulated with CO_2. All birds were set aside in the house and covered with a tarp. Three groups consisting of 17 birds were separated with groups 1 and 2 being composted, and group 3 held out of compost until the sampling was completed, all groups would be sampled similarly until incorporated into the compost pile. After the first day, all samples were pooled, consisting of five samples per tube, with their respective groups for both the tracheal and cloacal swabs. Group 1 was placed on the north side of compost pile and group 2 was placed on the south side of the compost pile after the third day of sampling. To maximize viable samples, groups 1 and 2 would be removed/replaced from compost for sampling on alternate days. The sampling of the groups was as follows: Birds from group 1 were sampled on day 1, 2, 3, and 4. Birds from group 2 were sampled on day 1, 2, 3, and 5. Birds from group 3 were sampled on day 1, 2, 3, 4, 5, and 6. Cloacal swabs were not performed on group 2 on day five due to decomposition, additionally only eight birds were sampled due to carcass integrity. On day six, only group 3 was sampled due to decomposition of group 1 and 2 carcasses. Carcass integrity limited the number of samples to eight birds from group 3, which were pooled into two tubes, four samples each, for both the tracheal and cloacal samples.

For environmental samples, plastic booties were worn while sampling feeders, waterers, and fan enclosures in the affected house. Samples were taken on day one, immediately following depopulation of the affected house. Feeders, waters, and fans were not available for sampling after the first day.

All swabs were placed in a broth media and placed on ice packs for transport to the California Animal Health and Food Safety Laboratory (CAHFS) for RT-PCR (4) and embryonated egg inoculation for virus viability assessments (5).

RESULTS

Average Ct values for tracheal samples on day 1 were 27.52, 27.69, 26.48 and 34.19, 33.13, 33.34 for cloacal swabs for the most clinically affected, clinically affected, and non-clinical birds, respectively. Environmental samples for litter, waterers, feeders, and fan enclosures had a Ct value of 31.05, 30.75, 30.83, and 31.60, respectively.

Day 2 and 3 results were pooled for all groups as composting had not begun. Average Ct values for tracheal swabs 24.11 and 24.49 and cloacal swabs 30.10 and 30.44 for day 2 and 3, respectively.

Day 4 group 3, non-composted, Ct values averaged 24.25 for tracheal samples and 30.23 for cloacal samples. Group 1, composted on the north side, had Ct values that averaged 25.20 and 29.70 for tracheal and cloacal samples.

Day 5 group 3, non-composted, Ct values averaged 21.73 for tracheal samples and 29.73 for cloacal samples. Group 2, composted on the south side, tracheal swab samples had Ct values that averaged 26.63.

Day 6, only group 3 was sampled with average Ct value of 25.02 and 33.28 for tracheal and cloacal swabs, respectively.

Virus isolation results are in progress and pending.

DISCUSSION

Previous studies have found that the high path H5N1 virus can survive in chicken feathers for up to 30 days when stored at temperatures at 20^{o}C and even longer when stored at 4^{o}C (6). However, during an event of an outbreak, poultry carcasses are covered and composted. Composting generates heat above 37^{o}C and is required to achieve 55^{o}C for a minimum of 72 hours in cases of HPAI when composting is the elected choice for carcass disposal (7). Currently, USDA-APHIS have a prolonged composting requirement during outbreaks with HPAI, with a minimum of 28 days. Based on virus viability in previous field studies that suggest the virus survives for only three days following euthanasia, the 28-day holding period, with an additional 14 days following removal of the composted material, would seem to be excessive and unnecessary. Furthermore, the additional holding time greatly hinders a business' continuity.

This study with the current data that is available highlights the importance of biosecurity during the first several days following depopulation of an infected premise. Virus particle can be found present within the carcasses for 6 days following euthanasia. However, virus particle and ability to infect are not necessarily one in the same and should be cautioned to use virus particles as a measurement of contamination. Should the virus isolation results from this study mirror the field study that was performed in 2022 (3), virus viability remains active for up to 72 hours following death of the bird. This would highlight the importance of a rapid response and enhanced biosecurity measures in the first 72 hours following depopulation of an infected premise.

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EXPLORING BROILER FIELD PERFORMANCE IN THE US (2020-2022): COMPARISON OF COCCIDIOSIS CONTROL STRATEGIES

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INTRODUCTION

Coccidiosis, an intestinal parasitic disease caused by *Eimeria*, poses great economic significance in the poultry industry. Due to the extended intestinal epithelial damage caused by *Eimeria* reproduction cycles, the physical integrity of the gut is damaged and symptoms such as diarrhea, dehydration, weight loss, and growth retardation in young chicks are accompanied. (1). Even without severe clinical symptoms, subclinical coccidiosis can cause economic loss by increasing mortality and reducing feed efficiency and the economic impact of coccidiosis has been calculated to exceed USD 3 billion annually (1, 2).

Two main approaches to prevent coccidiosis in the poultry industry are anticoccidial medications and vaccines. Anticoccidial drugs, used since the 1940s, can be chemical compounds or ionophores, but losing sensitivity in the field due to resistance has been a serious concern in the poultry industry (3). Additionally, rising concerns about antibiotic overuse have led to the adoption of programs like No Antibiotics Ever (NAE) or Antibiotic-Free (ABF). Ionophores are considered antibiotics in the U.S, leaving poultry producers with fewer options for anticoccidial drugs to combat coccidiosis.

In contrast to medication, vaccines offer an immunological approach. Live attenuated or unattenuated strains of multiple Eimeria species are used to induce protective immunity in chickens. Controlled exposure of chicks to *Eimeria* oocysts at the hatchery allows birds to develop *Eimeria* strain-specific immunity without experiencing clinical coccidiosis. Adequate oocyst reproduction in the chicks and reinfection from the litter is necessary (4) to establish uniform immunity, highlighting the crucial role of good farm management in successful vaccination against *Eimeria*.

While numerous attempts have been made to compare coccidiosis control programs, most studies conducted using battery cages or floor pens do not fully reflect the field situation. As a result, the implications of these study findings are often limited. In our previous study, one-year U.S broiler industry data was used to assess the impact of different coccidia control programs on broiler performance.

The findings highlighted the superior performance of broilers under the vaccine only program, compared to the chemical-only program, which exhibited subpar results. To validate the applicability of this finding, we extended our analysis over three years from 2020 to 2022. The blinded dataset provided by our industry partner included multiple performance indexes such as feed conversion ratio and flock mortality of complexes with various coccidiosis control programs.

MATERIAL AND METHODS

Data. The data analyzed in this study cover the period from January 2020 through December 2022. U.S. Broiler production information was provided by our industry partner, and any identifiable information, such as producer name, location, bird volume, weight volume, or plant number, was removed before the data was shared. The dataset included poultry production indexes collected monthly from an individual reporting unit: a broiler complex. A vertically integrated poultry producer in the U.S. consists of a production complex that includes a hatchery, a feed mill, a group of poultry growing farms, and a processing plant. In this study, we excluded coccidiosis control programs with mixed use of vaccination and anticoccidial medication from further analysis to reduce variability, specifically the bio-shuttle program and programs with both chemical compounds and ionophores. Additionally, only anticoccidial compounds in the starter and grower fees were considered when referring to the coccidiosis control program. Specific name of anticoccidial compound or vaccine used at each complex was not provided as it was considered identifiable information.

Thus, the data were sorted based on the general categories: chemical, ionophores, or vaccines. Coccidiosis control programs were named as follows: ionophore compounds in both the starter and the grower feed (ION-only), chemical compounds in both the starter and the grower feed (CHEM-only), and vaccination at the hatchery with no other anticoccidial

medication in the starter and grower feed (VAC-only). Anticoccidials that are a combination of an ionophore (narasin) and a chemical compound (nicarbazin), such as Maxiban® (Elanco), were classified as ionophores in this study.

For performance comparison, calorie conversion (adjusted to 6.7 lbs. weight), weekly, and total percentage mortality was assessed. In the dataset, market bird sizes ranged from 3.6 lbs. to > 8.5 lbs. For the study, the birds were divided into three weight ranges: less than 4.4 lbs. (< 4.4 lbs.), from 4.4 lbs. through 6.8 lbs. (4.4 – 6.8 lbs.), and more than 6.8 lbs. (> 6.8 lbs.).

Statistical analysis. Data were analyzed using Kruskal Wallis nonparametric analysis of variance and Dunn all pairs for joint ranks Post Hoc method with JMP software (version 16.2.0, SAS institute Inc. Cary, NC, 1989-2021). A significance level of $p < 0.01$ was considered.

RESULTS AND CONCLUSION

The total number of complexes included in this three-year dataset was 1653, comprising 7,598,074,234 heads of broilers for 2020, 1808 including 8,251,152,445 for 2021, and 1609, including 7,583,592,799 for 2022. According to USDA annual reports (5,6,7), this figure represented 70.5% of the 9,222,100,000 heads of broilers produced in the US during 2020, 90.3% of 9,130,700,000 during 2021, and 82.7% of 9,165,400,000 during 2022. Throughout the three-year period, the number of complexes treated with ION-only or CHEM-only programs gradually decreased from 13% in 2020, 10% in 2021, and 9% in 2022. Simultaneously, the number of complexes with CHEM-only programs experienced a rapid drop from 47% in 2020 to 30% in 2021, only to rise again to 36% in 2022. The usage of the VAC-only program gradually increased from 18% in 2020 to 25% in 2022. Bio-shuttle programs, starting with vaccination followed by CHEM or ION during the growth period, represented 22% in 2020, increased to 39% in 2021, and decreased in 2022 to 29%. Bio-shuttle programs were excluded from further analysis in this study.

The first-week mortality and cumulative mortality at 42 days of age were compared each year (data not shown). There was an increasing trend in first-week mortality over the three years, starting from the mean (±S. D) of 1.57% (± 0.55) in 2020 to 1.69% (±0.63) in 2021, and 1.78% (±0.69) in 2022. A similar trend was observed in cumulative mortality at 42 days of age, beginning with 4.14% (±1.16) in 2020, 4.46% (±1.23) in 2021, and 4.72% (±1.58) in 2022. The 1st week mortality (data not presented) and the cumulative mortality at 42 days of age (Figure 1) were compared across the different programs. Each program was distinguished by a specific color: blue for the CHEM-only program, red for the ION-only program, and green for the VAC-only program. Across all three years, birds following the VAC-only program exhibited significantly lower first-week mortality and cumulative mortality at 42 days of age compared to those in the CHEM-only programs. When contrasted with the ION-only programs, birds in the VAC-only programs demonstrated significantly lower first-week mortality across all three years.

Regarding cumulative mortality at 42 days of age, both ION-only and VAC-only programs yielded comparable results, except in 2022 when VAC-only treated birds exhibited significantly lower mortality compared to the ION-only programs. Adjusted calorie conversion (adjusted to 6.7 lbs. weight) serves as a measure of feed efficiency. In our previous study, we found no significant difference in feed efficiency for small birds weighing less than 4.4 lbs among the three programs, and this trend persisted in 2021 and 2022 as well (data not shown). For birds weighing between 4.4 lbs and 6.8 lbs, as well as large birds weighing more than 6.8 lbs, the VAC-only program exhibited either equal or significantly better feed efficiency compared to the other coccidiosis programs in the study (Figure 2 A and B).

In this study, the US broiler production data from 2020 to 2022 were analyzed to examine the performance trend of birds under different coccidiosis control programs. Year by year, overall chick mortality exhibited an upward trend from 2020 to 2022, possibly associated with the pandemic period and industry-wide chick quality issues (8). The results from our prior study indicated that birds treated with the CHEM-only program had statistically higher total live production costs and weekly and total percentage mortality compared to those treated with the ION-only or VAC-only program. Additionally, birds on the VAC-only program demonstrated comparable feed efficiency, as represented by adjusted calorie conversion, to other programs.

Utilizing three years of data, we confirmed that the chemical coccidiosis program does not outperform the vaccine-only program concerning both mortality and feed efficiency. Despite intrinsic issues in the data related to limited information on confounding factors, such as climate conditions, medical and management history, and exact anticoccidial medication, the substantial sample size included in this analysis provides a comprehensive overview of the performance comparison among different coccidiosis control programs.

In conclusion, the multi-year analysis of broiler performance in the United States from 2020 to 2022 consistently underscores the superior effectiveness of the vaccine-only program. These findings challenge

conventional notions and highlight a paradigm shift in coccidiosis control strategies. The observed trends in decreasing adoption of ionophore and chemical programs, coupled with an increasing embrace of vaccination, suggest a growing industry recognition of its long-term benefits. The study emphasizes the need for continued research and adaptation, advocating for a reassessment of prevalent beliefs in poultry health management.

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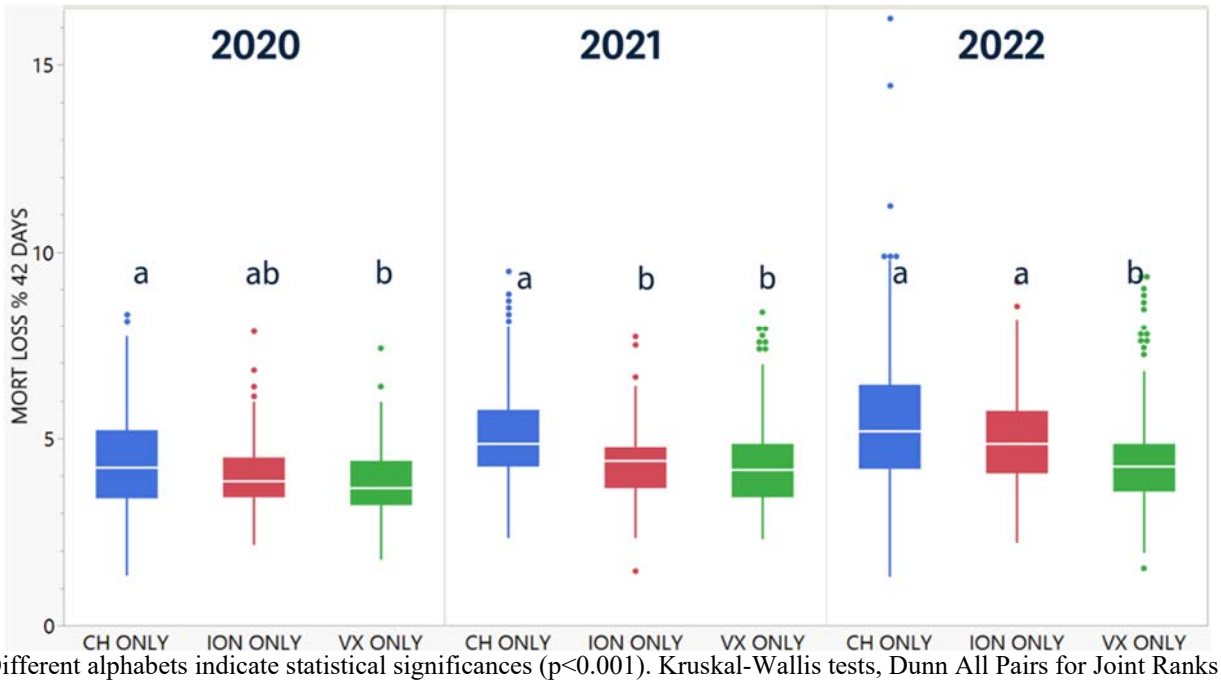
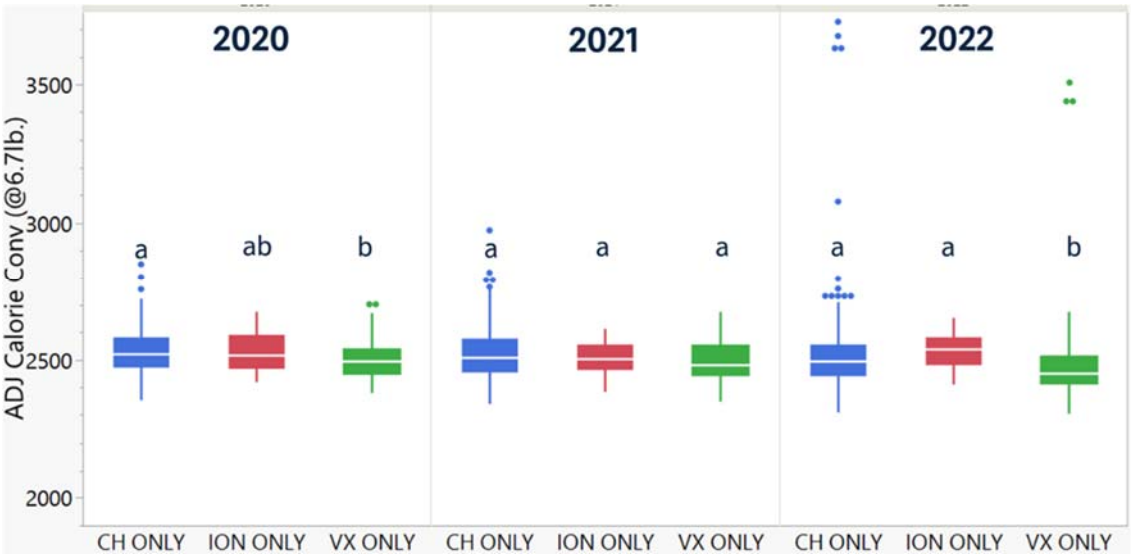
Figure 1. Cumulative mortality at 42 days of age of each cocci program.

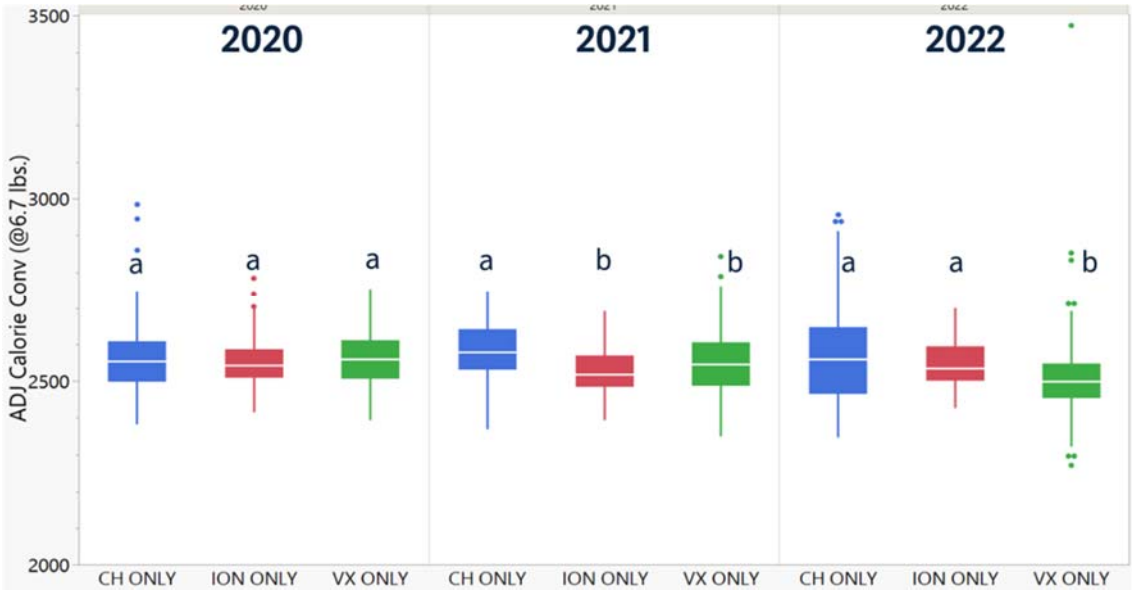


Different alphabets indicate statistical significances ($p<0.001$). Kruskal-Wallis tests, Dunn All Pairs for Joint Ranks
Figure 2. Comparison of Adjusted calorie conversion (at 6.7 lbs) for birds between 4.4 lbs and 6.8 lbs (A) and birds more than 6.8 lbs (B).

A.



B



Different alphabets indicate statistical significances ($p<0.001$). Kruskal-Wallis tests, Dunn All Pairs for Joint Ranks

EFFICACY OF A RECOMBINANT HVT-IBD-ND VACCINE AGAINST CHALLENGE WITH VERY VIRULENT IBDV, VELOGENIC NDV, AND VIRULENT MDV IN BROILER BIRDS WITH MATERNAL ANTIBODIES

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SUMMARY

A recombinant HVT-IBD-ND dual insert vaccine was developed for protection against infectious bursal disease (IBD), Newcastle disease (ND) and Marek's disease (MD). The objective of the following four studies was to evaluate efficacy provided by HVT-IBD-ND vaccination against IBD, ND and MD in commercial broilers with maternal antibodies. In the first two studies, commercial broilers were vaccinated in ovo (E18) or subcutaneously (SC) on day of hatch (Day 0) and challenged with a very virulent IBDV on Day 35 or 49, respectively. For the in ovo vaccination study that was challenged on Day 35, 67% (16/24) of the birds were protected for the vaccinated group, compared to 13% (3/24) for the control group (P value=0.0005). For the subcutaneously vaccinated study that was challenged on Day 49, 87% (20/23) of the birds were protected, compared to 0% (0/24) for the control group (P value=0.0003). The third study was conducted to examine ND efficacy. Commercial broilers were vaccinated either in ovo, or subcutaneously on day of hatch. On Day 34, birds were challenged with a velogenic NDV Herts Weybridge 33/56. Protection of 100% (30/30) was observed for both in ovo and subcutaneously vaccinated groups while 0% (0/30) protection was observed for the control group. The fourth study was conducted to examine MD efficacy. Commercial broilers were vaccinated either in ovo, or subcutaneously on day of hatch. On Day 11, birds were challenged with a virulent MDV GA22. The *in ovo* and SC vaccinated groups were 61% (49/80) and 57% (47/82) protected, respectively, compared to the control group with 18% (14/78) protection (P value <0.0001 for either group).

INTRODUCTION

Infectious bursal disease (IBD) is an acute and highly contagious viral infection of young chickens that causes immunosuppression and increased susceptibility to other infectious agents. Newcastle disease (ND) is a highly contagious and fatal disease affecting all species of birds. Marek's disease (MD) is a common cause of condemnations and immune suppression in broilers. A recombinant HVT-IBD-ND tri-valent vaccine was developed. The efficacy of the vaccine in broilers with maternal antibodies was investigated in vaccination/challenge studies.

MATERIALS AND METHODS

HVT-IBD-ND vaccine (Poulvac® Procerta™ HVT-IBD-ND): HVT-IBD-ND is a recombinant viral vaccine. IBDV VP2 and NDV F gene expression cassettes were inserted into the HVT genome. 0.05 mL/egg (*in ovo*), 0.2 mL/bird (subcutaneous injection).

Challenge viruses. Very virulent (vv) IBDV California strain; velogenic NDV Herts Weybridge 33/56; virulent MDV GA22

Broiler birds. Commercial broilers (Allens/Lohmann VALO)

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

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

IBDV/NDV/HVT serology. IDEXX IBD ELISA; BioCheck ND F ELISA; HVT IFA GMT (geomean titer); nd (not done)

Efficacy assessment.

vvIBD: Mortality, clinical signs, histological lesion scores, 10 days post challenge

velogenic ND: Mortality, clinical signs, 11 days post challenge

virulent MD: Mortality, clinical signs, gross lesion, 70 days post challenge

Statistical analysis. Comparison was made between the non-vaccinated challenged and each vaccinated challenged treatment. All hypothesis tests were at the $p \leq 0.05$ level of significance.

RESULTS

Commercial broilers with MDA of 7703 (IBD ELISA titer) were vaccinated with HVT-IBD-ND in ovo. Challenge with a vv IBDV was performed on Day 35 and bursal histology lesion was scored on Day 45. Sixty-seven % protection (16/24) was observed for the vaccinated birds while the challenge control birds had 13% protection (3/24). In another study, commercial broilers with MDA of 5663 (IBD ELISA titer) were vaccinated with HVT-IBD-ND subcutaneously. Challenge with a vv IBDV was performed on Day 49 and bursal histology lesion was scored on Day 59. Eighty-seven % protection (20/23) was observed for the vaccinated birds while the challenge control birds had 0% protection (0/24).

Commercial broilers with MDA of 16084 and 14274 (NDV F ELISA titers) were vaccinated with HVT-IBD-ND in ovo or subcutaneously. A velogenic NDV challenge was performed on Day 35. One hundred % protection (30/30) was observed for either vaccinated group while challenge control had 0% protection (0/30).

Commercial broilers with MDA of 256 (HVT IFA titer) were vaccinated with HVT-IBD-ND in ovo or subcutaneously. Challenge with a virulent MDV was performed on Day 11. Sixty-one % protection (49/80) was observed for the in ovo vaccinated group and 57% (47/82) was observed for the subcutaneously vaccinated group. The challenge control had 18% protection (14/78).

DISCUSSION/CONCLUSION

67% protection was observed against a very virulent IBDV challenge on Day 35 for commercial broilers vaccinated in ovo (E18) with HVT-IBD-ND, while unvaccinated control broilers had 13% protection, showing statistical significance ($P=0.0005$). 87% protection was observed against a very virulent IBDV challenge on Day 49 for commercial broilers vaccinated subcutaneously on day of hatch with HVT-IBD-ND, while unvaccinated control broilers had 0% protection, showing statistical significance ($P=0.0003$).

100% protection was observed against a velogenic NDV challenge on Day 35 for commercial broilers vaccinated in ovo or subcutaneously with HVT-IBD-ND, while unvaccinated control broilers had 0% protection.

61% and 57% protection were observed against a virulent MDV challenge on Day 11 for commercial broilers vaccinated with HVT-IBD-ND in ovo or subcutaneously with HVT-IBD-ND, respectively. The unvaccinated control broilers had 18% protection, showing statistical significance ($P<0.0001$) for either group.

ACKNOWLEDGEMENTS

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

Tables.

Trt	Vaccine	Route of Vaccination	IBDV Serology ELISA (% Pos/GMT)				% vvIBD Efficacy		
			D0	D14	D26	D34	% Mortality	% Lesion ≥3	% Protection
T01	Non-vaccinated	NA	100 (7841)	93 (2033)	45 (106)	33 (83)	4 (1/24)	87 (20/23)	**13 (3/24)**
T03	HVT-IBD-ND	In ovo	100 (7703)	86 (1699)	37 (89)	22 (138)	**0** (0/24)	33 (8/24)	**67 (16/24)**
							T01 vs. T03: P=0.0005		

Trt	Vaccine	Route of Vaccination	IBDV Serology ELISA (% Pos/GMT)				% vvIBD Efficacy		
			D0	D26	D41	D48	% Mortality	% Lesion ≥3	% Protection
T01	Non-vaccinated	NA	100 (5663)	25 (114)	12 (57)	4 (18)	0 (0/24)	100 (24/24)	**0 (0/24)**
T03	HVT-IBD-ND	Subcutaneous	nd	48 (290)	70 (518)	85 (941)	0 (0/23)	13 (3/23)	**87 (20/23)**
							T01 vs. T03: P=0.0003		

Trt	Vaccine	Route of Vaccination	% Hatch	NDV F Serology ELISA (% Pos/GMT)			% ND Protection
				D0	D28	D34	
T02	Non-vaccinated	NA	85 (68/80)	100 (21454)	0 (189)	0 (157)	**0 (0/30)**
T03	HVT-IBD-ND	In ovo	92 (74/80)	100 (16084)	90 (24668)	100 (9781)	**100 (30/30)**
T04		subcutaneous	95 (76/80)	100 (14274)	90 (4976)	100 (14544)	**100 (30/30)**

Trt	Vaccine	Route of Vaccination	HVT Serology IFA (% Pos/GMT)		% MD Efficacy		
			D0	D10	% Mortality/ Clinical Signs	% MD Lesions	% Protection
T01	Non-vaccinated	-	100 (256)	100 (220)	63 (49/78)	73 (57/78)	**18 (14/78)**
T02	HVT-IBD-ND	In ovo	100 (256)	90 (128)	23 (18/80)	26 (21/80)	**61 (49/80)**
T03		subcutaneous	100 (239)	100 (235)	30 (25/83)	29 (24/82)	**57 (47/82)**
					T01 vs. T02: P <0.0001; T01 vs. T03: P <0.0001		

FIELD EVALUATION OF THE EFFICACY OF LIVE *E. COLI* AND LIVE *SALMONELLA* TYPHIMURIUM VACCINATION BY PARENTERAL ADMINISTRATION IN COMMERCIAL LAYERS

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INTRODUCTION

Live *E. coli* and *Salmonella* Typhimurium (ST) vaccines have been used in the commercial layer industry for almost two decades to help reduce mortality from colibacillosis and aid in the control of *Salmonella*, which are some of the main concerns in the egg industry. Currently, these live vaccines are only applied by mucosal application, either coarse spray or drinking water. However, due to their unique attenuation properties, recent controlled studies have shown that these vaccines may provide additional benefits when administered parenterally. This presentation will summarize the results of two field trials where live *E. coli* and ST vaccines were administered parenterally in caged and cage-free commercial egg-type pullets. The parenteral application's safety, protection, and serological response will be evaluated and compared to the conventional application methods.

MATERIALS AND METHODS

In Study 1, 54,000 Leghorn-type pullets were placed in a barn with four rows of conventional cages. The birds were equally divided between the four rows and were raised under the same management and nutritional conditions. The birds in the first three rows (Control, 40,500 pullets) received a typical vaccination program that included a live *E. coli* (LVEC) vaccine by coarse spray on weeks 5, 8, and 16. The birds placed in the fourth row (Treatment, 13,500 pullets) received the LVEC vaccine by coarse spray on weeks 5 and 8, and instead of getting the third dose via spray, the vaccine was mixed with a commercial 3-way killed vaccine and applied intramuscularly in the breast at 12 weeks of age. Each group's mortality, body weights, and uniformity were recorded and compared during rearing and production until the flock finished its productive cycle at 75 weeks. Health evaluations of each group were performed at weeks 31 and 52.

In Study 2, 53,000 brown pullets were placed in a cage-free aviary system. All the birds were vaccinated with a LVEC vaccine on day 2 by coarse spray and a live ST (LVST) vaccine on day 1, week 2, and week 6 by coarse spray. At 10 weeks of age, the birds received a 3-way SE-ND-IB killed vaccine intramuscularly and were divided into four groups: a) Control group – Killed vaccine only; b) Group 1 – Killed vaccine + LVEC; c) Group 2 – Killed vaccine + LVST; d) Group 3 – Killed vaccine + LVEC + LVST. Each group's mortality, body weights, and uniformity were recorded and compared during rearing and production. Each group was bled for Group B/D *Salmonella* ELISA titers at weeks 14, 34, and 54. Health evaluations of each group were performed at weeks 29 and 47.

RESULTS

In Study 1, there were no significant differences in body weights and uniformity between the two groups in both rearing and production. Mortality was significantly lower in Group 2 during rearing, but no significant differences were observed during production. Overall, both groups met the production and mortality targets for the breed during rearing and production. None of the two groups showed lesions consistent with colibacillosis and/or severe vaccine reactions on the health evaluations.

In Study 2, there were no significant differences in body weights, uniformity, and mortality between groups in both rearing and production (up to week 48, when this proceeding was submitted). Mortality in all groups was within target for the breed during rearing, but it was elevated during production (up to week 48). However, mortality in this flock was lower than what is usual in this farm. None of the groups showed lesions consistent with colibacillosis and/or severe vaccine reactions on the health evaluations. At week 18, the *Salmonella* B/D titers were 220% higher in Group 3 and 78% higher in Group 4 than the controls and Group 1 titers, which did not receive the LVST vaccine intramuscularly. Similarly, at week 34, the *Salmonella* B/D titers were 280% higher in Group 3 and 320% higher in Group 4 than the controls and Group 1 titers. *Salmonella* B/D serology will be performed again at weeks 54 and 74.

DISCUSSION

The two field studies summarized in this proceeding demonstrated the safety of applying a LVEC and LVST vaccine intramuscularly, either alone or in combination, mixed with a 3-way killed vaccine, in healthy white and brown commercial pullets. The birds that received the live vaccines intramuscularly did not show any differences in mortality, body weights, and uniformity during rearing when compared to birds that received the vaccines by a conventional application method and they were on target for their respective breed standard. Additionally, no severe vaccine reactions in the pectoral muscle were observed.

In Study 1, there were no significant differences in mortality between the control and the treatment groups and the flock performance was above standard in both rearing and production. The posting sessions showed that there was not a significant *E. coli* challenge in this flock, which could be the reason why there were no differences in mortality between the two groups.

Similarly, in study 2, there were no significant differences in mortality, body weight, and uniformity between groups in rearing and production (up to week 48). It still needs to be determined if the LVEC parenteral application will provide better and longer protection against colibacillosis in this study, and since this trial is still ongoing, more data will be available at the time of the presentation. However, the *Salmonella* B/D titers were significantly higher at weeks 18 and 34 in the groups that received the LVST vaccine intramuscularly than in the groups that did not. This finding aligns with what has been observed in previous controlled studies, in which the application of a LVST vaccine parenterally elicited a stronger serological response, perhaps due to the exposure of much more vaccine to circulating mononuclear cells.

In conclusion, these studies showed that it is safe to administer the LVEC and LVST vaccines intramuscularly in white and brown commercial pullets. They also showed the potential of improving *E. coli* and *Salmonella* vaccine protection by working differently with the tools that are already in existence.

UTILIZATION OF POSTBIOTICS TO HELP PROMOTE GUT HEALTH IN POULTRY PRODUCTION

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SUMMARY

Viable alternatives to improving gut health have increased over the last decades as the concerns and demands for reduction in antibiotic usage continue to rise. Supplementation with prebiotics, probiotics and postbiotics have provided a more naturally safe alternative to antibiotics and have shown to be effective in improving health, livability, and production performance in poultry production systems. Postbiotics have been known to improve gut health through promotion of competitive exclusion, improving gastrointestinal villi development, stimulating natural immunity, and have antioxidant action among other beneficial effects. Since postbiotics are a byproduct of living organisms and are not alive, they are able to still provide their therapeutic applications while withstanding the stressors of the feed manufacturing process. This property makes their use very advantageous to certain areas of the poultry industry. Therefore, in the era of alternative beneficial products, we review the potential of postbiotics as another tool in improving overall gut health.

POULTRY INDUSTRY CHANGES AND CHALLENGES TO INTESTINAL HEALTH

As the consumption of poultry continues to increase globally, the demand for meat and eggs that are produced without antibiotics while still maintaining environmental responsibility has also increased. Poultry meat is typically viewed as a healthier choice; however, antimicrobial resistance reports continue to spotlight the agricultural sector as one of the leading areas of concern (1). This growing concern has led some countries to intensely regulate the use of antibiotics and/or ban antibiotics used for growth promotion (2). Due to the intense nature of animal rearing practices, confined environments, and lack of efficacious treatment options, pressures from viruses, bacterial and protozoal agents have increased; particularly for systems that have limited antibiotic usage such as no antibiotics ever, antibiotics free, no antibiotics important to human medicine and organic (3).

In poultry, a correlation between a healthy gastrointestinal (GI) system and profitability has driven the industry to pay closer attention to maintaining and improving the integrity of the gastrointestinal tract (4). When the functional aspects of the gastrointestinal tract (GIT) such as its physical, chemical, microbiological or immunological components become negatively affected, their balance is also impacted. Factors such as diseases, stressful environmental conditions, lower quality or deficient diets, and poor management can result in GI condition changes. As a result, feed efficiency losses can be measured through increased feed conversion ratios, decreased livability, lower bird weights and overall decreased productivity (5). Other parameters that are impacted by GI imbalance are food safety, environmental, welfare and overall bird health (5).

Pathogenic viruses, bacteria and parasites can negatively impact the GIT locally and systemically leading to decreased digestion and absorption of nutrients (6). Locally, pathogens can cause inflammation, oxidative stress, and the physical barrier function of the gastrointestinal wall may be affected leading to “leaky” gut syndrome (7, 8). The chemical composition, and the normal microbiota populations can also be hindered. Pathogens that have an effect enterically can also exert an effect systemically involving other organs and systems; hence, adding to the stress of an already compromised host. Conversely, pathogens that affect other organs and systems can also lead to GI disruption. One area of concern associated with a disruption of the balance of GIT is dysbiosis (9). The microbiota of the GIT of the bird plays a key role in preventing other pathogens from colonizing, participates in immune system communication through signaling and regulatory pathways and help provide nutrition to the bird by breaking down nutrients and secrete metabolites that provide energy to enteric epithelial cells(6).

While there are many benefits important to maintaining a healthy GIT, gut health continues to be one of the most challenging areas to manage. Therefore, the poultry industry has increased interest in solutions and alternatives that aid the GIT and provide benefits to the microbiota.

THE USE OF PREBIOTICS, PROBIOTICS, AND POSTBIOTICS IN POULTRY

The use of antibiotics for growth promotion (AGP) purposes was a common practice that helped reduce mortality and improve the feed efficiency in the poultry industry (10). It was hypothesized that their effect was on reduction of pathogenic bacteria in the GIT of the bird. However, conflicting evidence shows variability in findings showing no difference on performance and potential contribution to antibiotic resistant pathogens such as *Salmonella* and *Campylobacter* (11, 12). Microbiome research along with newer techniques for metagenomic sequencing have allowed for faster characterization of microbiota populations as well as their metabolites and interactions (12-14). These findings paved way to increased interest in alternatives tools to AGP's such as prebiotics, probiotics and postbiotics.

Probiotics are live microorganisms that exert a health benefit on the host when consumed (15). Over the past five decades, the understanding and usage of probiotics in the agricultural sector has increased due to the positive properties attributed (16). Their benefits include improvements in host immunity, digestion of nutrients, structural integrity and function, production of organic acids, molecules, and enzymes as well as pathogen colonization exclusion and interactions with other GI microbes (17, 18). Many studies have shown a positive correlation on the use of probiotics in poultry improving body weight gains (BWG) and feed conversion ratios (FCR), while few studies have shown no beneficial effects or a negative effect(16, 18-21). Variability of effectiveness and desired levels of benefits are dependent on the species of microbe as well as their shelf life and survivability in the feed manufacturing process (22).

Prebiotics are predominantly carbohydrate-based substances and derivatives that are known to have an overall beneficial effect on the host and the commensal bacterial communities of the bird's GIT, particularly in the ceca (15, 23). Benefits such as improved performance parameters have been reported in poultry (21). Prebiotics also have an effect in immunomodulation, mineral absorption, pathogen control and other metabolic functions (24). Indirect and direct effects have been described with prebiotics use where commensal microbiota can metabolize prebiotics into metabolites such as short chain fatty acids (SCFAs) that have benefits to the host while at the same time providing benefits to other commensal bacteria. Prebiotics have also been recognized to play a role in reduction of pathogenic bacteria through competitive exclusion and prevention of attachment of bacterial toxins to the intestinal epithelium (25). Prebiotics and probiotics have provided a viable alternative to AGPs provided the many benefits as part of a microbiota management tool (24).

Other biotics such as synbiotics and postbiotics have also been described as having beneficial effects on the host and microbiota (26). Synbiotics is a combination of living microorganisms and substrate(s) that have a beneficial effect on the host. The benefits of synbiotics have shown positive effects on growth parameters, feed efficiency, immunomodulation and being an alternative to AGPs (27). Postbiotics are emerging as another valuable tool to promote bird health and improve performance capabilities. They have been defined as a beneficial preparation of non-living microorganisms and/or their components (28). Metabolic products of postbiotics such as SCFAs, peptides, proteins, carbohydrates and bacteriocins offer another tool for improving poultry production and a viable alternative to AGPs (29).

A CLOSER LOOK AT POSTBIOTICS AND POULTRY GUT HEALTH

Postbiotics offer a wide range of health benefits to the host including anti-inflammatory, antioxidant, antibacterial and anti-proliferative properties (30, 31). Cell wall derivatives of *Lactobacilli* and *Bifidobacterium* and known to have immunomodulatory effects through cell mediated and humoral immune responses (32). Previous studies have shown anti-inflammatory effects from *Lactobacilli* spp. by suppressing production of proinflammatory cytokines (31, 32). Prolonged inflammation, particularly in the GIT of poultry can lead to increased morbidity and mortality (5). One way that postbiotics exert anti-inflammatory effects is through modulation of the kappa-light-chain-enhancer of activated B cell (NF-κB) and/or mitogen-activated kinase (MAPK) pathways (33, 34). Some postbiotic components of *Lactobacilli* strains have been reported to positively influence the GIT barrier and improve the protection against infections (34). This response is due to increases in production of specific heat shock proteins and antimicrobial peptides (35).

While derivatives of inactivated bacterial cultures tend to be most popular, postbiotic derivatives from yeast such as *Saccharomyces cerevisiae* can also generate highly beneficial postbiotics (36). Reports of increased BWG and improved FCR have been observed across various studies (37). This improvement could be attributed to the improvements in villi length, crypt depth, and villi length to crypt depth ratios which lead to improvement of nutrient absorption. Another beneficial effect of postbiotic derivatives fermentation product of Saccharomyces cerevisiae is inhibition of pathogenic bacteria such as *Salmonella*, *Enterococcus*, and *E. coli* (38, 39). These

preparations help reduce the pH level of the gut and discourage the growth of pathogenic harmful bacteria. Another benefit of postbiotics has been described in mitigation of mycotoxins. Mycotoxins are known to be immunosuppressive and can also affect the GIT resulting in reduced productivity. Preparations of postbiotic yeast cell wall extracts were able to limit toxin absorption and decrease the effects of deoxynivalenol (DON), T-2 toxin (T2) and zearalenone (ZEA) (40).

ADVANTAGES AND DISADVANTAGES OF POSTBIOTICS

One main advantage of postbiotics over probiotics is that they are non-living. This implies that they can undergo the pelleting process, withstand higher amounts of heat and have longer shelf life (29). They are still able to provide the health benefits needed to improve performance parameters, reduce morbidity and mortality, and reduce the use of AGPs. As an antibiotic alternative, postbiotics offer another indirect advantage as their use may be better accepted by consumers (41, 42). While postbiotics are generally considered safe, one concern is the potential of antimicrobial resistance gene transference (19). The industry has yet to understand the potential of postbiotics as all their properties and interactions may not be well known.

A disadvantage of postbiotics is that they must be derived from the same consistent defined microorganisms and processing and extraction must be well stablished. Any variability in the process may lead to inconsistent metabolites and/or variable reproducibility of responses (29). Regulatory authorities such as the FDA have not addressed postbiotics; however, they may address it in the future to define their usage, safety, and efficacy (28). Postbiotics don't have living organisms and they have yet to have defined regulatory practices, therefore product developers are able to maintain ownership of their ingredients. While this is considered an advantage, it can also be a disadvantage because of the difficulty of reproducing their effects whether beneficial or not by other researchers or institutions (28).

CONCLUSIONS

Postbiotics have offered yet another viable solution to promote gut health in poultry production. They have an indirect and a direct effect on the bird's immunity, performance parameters, and overall health. Postbiotics have a wide spectrum and offer multiple benefits; however, they may not always be repeated consistently. In the era of reduction of antibiotic use, postbiotics are another tool in the biotics toolbox. Therefore, as we continue to look for alternatives to AGPs and/or complementing conventional programs in gut health, further research may enhance our knowledge on the broad applications of postbiotics.

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THE THYMUS: A KEY IMMUNOLOGICAL ORGAN BUT OFTEN IGNORED IN THE DIAGNOSIS OF AVIAN DISEASES

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SUMMARY

The development of the avian immune system depends on the bursa of Fabricius (BF) and thymus as the source of B and T lymphocytes, while bone marrow is the source of macrophages and heterophils. All three organs are susceptible to infections which influence immune responses. Postmortem examination of the BF is routinely done in a diagnostic laboratory because infectious bursal disease virus causes destruction of B lymphocytes affecting the humoral immune response. In contrast, the thymus is often ignored during the postmortem examination, while virus infections, e.g., chicken anemia virus a member of the Gyrovirus genus in the *Anelloviridae*, can cause considerable damage to the thymus affecting cytotoxic and helper T cells. In this review, the normal structure of the thymus and methods to evaluate the damage are discussed. In addition, the impact of viruses, including newly discovered cycloviruses and gyroviruses, replicating in the thymus are also discussed.

INTRODUCTION

The proper diagnosis of any disease in poultry submitted to a diagnostic laboratory or during a visit to a farm requires a complete and careful investigation of all organs in the diseased animals including the complete lymphoid system. The bursa of Fabricius (BF) is routinely included in the examination of poultry when appropriate based on the age of a flock with problems. Interestingly, there is frequently silence when I asked at meetings with diagnosticians, including the Western Poultry Disease Conference in 2023, *"What about the thymus?"*

In this paper, I will briefly mention the primary and secondary immunological organs in chickens, followed by providing an overview of known pathogens causing damage to the primary immunological systems especially the thymus and resulting in immunosuppression. Finally, I will discuss several recently described pathogens affecting the thymus and the need to use advanced diagnostic techniques to find additional pathogens that may cause immunosuppression. However, it needs to be emphasized that the most advanced diagnostic approaches cannot replace a complete postmortem examination that includes the thymus.

PRIMARY AND SECONDARY IMMUNOLOGICAL ORGANS IN CHICKENS

The thymus, BF, and bone marrow, referred to as the primary lymphoid organs, are the source of T cells, B cells and macrophages/dendritic cells, respectively. The secondary lymphoid tissues consist of the spleen and the many mucosal-associated tissues, e.g., cecal tonsils and the Harderian gland. The development of secondary lymphoid tissues is partly antigen driven. The anatomy of the primary and secondary lymphoid organs has been described in detail by Nagy *et al*. (1). The avian thymus consists of two rows of 7 to 8 lobes located in the neck region parallel to the vagus nerve and the jugular veins. Most lobes are easily visible when the skin is opened along the ventral side of the neck. Each lobe consists of a cortex and a medulla and is surrounded by a fibrous capsule. The cortex consists of precursor $CD3^+CD4^+CD8^+$ T cells, which migrate towards the medulla before entering the bloodstream and secondary lymphoid tissue as $CD4^+CD8^-$ and CD^-CD8^+ T cells. Relevant in the context of this paper is the high rate of cell division in the thymus as will be explained in the section on immunosuppressive viruses. The $CD4^+CD8^-$ T helper (Th) cells are important for the development of the switch from IgM to IgY and IgA after antigenic stimulation. The CD^-CD8^+ cytotoxic T cells (CTL) are important for the control of intracellular pathogens. Detailed information on the ontogeny and functional maturation can be found in several chapters of the third edition of Avian Immunology (2). The practical take-home message is that by ignoring the examination of the thymus, one can miss important information regarding the cell-mediated and humoral immune responses after infections or vaccinations. The lack of Th cells will negatively impact antibody responses to pathogens and vaccines even if the BF is fully functional.

In contrast with the thymus, the BF is included routinely in a post-mortem examination. Most poultry diagnosticians are familiar with the histology of the normal BF, which is detailed in different text books (e.g., 2-4). Thymus and BF are undergoing involution

starting between 3 and 4 months of age and are no longer present in adult chickens unless tumors have developed. For example, lymphoid leukosis caused by avian leukosis virus subgroup A and B starts with tumors in the BF.

The bone marrow is the third organ imported for the development of immune responses to pathogens. Hematopoietic stem cells are the source for erythrocytes, thrombocytes, heterophils, macrophages and dendritic cells (1). The latter two are important for antigen processing and presentation of antigens to T and B lymphocytes. Heterophils, macrophages and thrombocytes are phagocytosing cells needed for the control of bacterial infections. The development of these cells from the stem cells to mature cells requires continuous cell divisions.

IMMUNOSUPPRESSION

The following definition of immunosuppression is used in this paper: "A state of temporary or permanent dysfunction of the immune system and leading to increased susceptibility to disease and often with a suboptimal innate, antibody and cell-mediated immune responses" (5-8). Many pathogens can cause one or more of these effects but often in combination with clinical disease. In those cases, the primary diagnosis is based on the pathological findings during the post-mortem examination. In this section, I will discuss subclinical immunosuppression in the absence of clinical disease with an emphasis on the thymus.

The most important viruses affecting the thymus are Marek's disease virus (MDV) prior to developing clinical disease and chicken infectious anemia virus (CIAV). Infection with MDV may cause severe thymus and bursa atrophy (TA and BA, respectively) during the early cytolytic infection phase (9) but proper vaccination prevents the severe cytolytic infection. In addition, so-called late immunosuppression has been described by Dr. Gimeno and her team (reviewed in 8). Properly vaccinated chickens infected with very virulent plus (vv+) strains of MDV and vaccinated against infectious laryngotracheitis virus (ILTV) showed a reduced protection against ILTV in the absence of lymphomas. Diagnosis of MDV as the causative agent in TA and BA can be made by immunohistochemistry on sections of the thymus and/or BF using monoclonal antibodies against MDV glycoprotein B or pp38. Identifying MDV as the culprit in the so-called late immunosuppression" remains problematic in the absence of clinical MD because the mechanisms responsible for these breaks have not been identified to my knowledge.

The genome of CIAV consists of single-stranded, negative sense, covalently linked DNA. To replicate the virus the genome forms a double-stranded circular DNA like a small plasmid. Infection with CIAV requires dividing cells for viral replication because the small viral genome of 2.3 kb lacks the enzymes to make viral DNA. Dividing cells provide the necessary enzymes not only to reproduce its own DNA but also the genome of small DNA viruses. Hemocytoblasts in the bone marrow, thymocytes and dividing Th cells and CTL responding to infections or vaccinations are the key target cells for CIAV. Infection can cause severe TA after maternal antibodies have waned especially if antibody responses are suboptimal after infection with infectious bursal disease virus (IBDV) or even after vaccination with "hot" IBDV vaccine strains. Figure 1.A shows normal histology of the thymus in a two-week-old broiler chicken and Figure 1.B shows atrophy of the thymus cortex associated with CIAV infection. An important aspect of CIAV infection is the ability of the virus to remain present in gonads and other organs in the absence or presence of virus-neutralizing antibodies. It has not been elucidated if this form of latency is based on the presence of double-stranded circular DNA like a small plasmid, virus particles or both. Diagnosis of CIAV infection is based on the detection of viral DNA using real-time quantitative (q)PCR assays. High virus levels as indicated by low threshold cycle (Ct) values, in general values between 25 to 35, are suggestive of a current or recent infection. Low levels of viral DNA (high Ct values close to 40) may reflect a latent infection. Real-time qPCR assays for the detection of actively replicating virus require viral RNA (quantitative-reverse transcription (qRT)-PCR assays, which have been described (10, 11). Unfortunately, very few laboratories have standardized the qRT-PCR assay for CIAV. Detection of antibodies using Elisa kits indicates that the birds had an infection or were vaccinated at some point in time.

Limited data suggest that reovirus, adenovirus, avian leukosis virus and reticuloendotheliosis virus may also cause immunosuppression which in the case of reovirus has shown to impact the thymus (reviewed in 7).

It is important to realize that not all cases of subclinical immunosuppression are caused by pathogens. For example, stress-induced corticosterone can cause rapid decreases in lymphoid cells in the thymus and BF (12). Inhibition of DNA, RNA and or protein synthesis will cause immunosuppression if rapidly dividing cells in the thymus, BF or bone marrow are affected. This mechanism has been proposed for some of the mycotoxins such as *Fusarium*-derived deoxynivalenol (DON) (reviewed in 13, 14).

NEWLY DISCOVERED DNA VIRUSES CAUSING IMMUNOSUPPRESSION

Over the last 15 years, it has become clear that there are many viruses with a small, single-stranded, covalently linked DNA genome. Two virus families are potentially important for poultry. The *Circoviridae* contain two genera: Circovirus and Cyclovirus (15). The *Anelloviridae* have also two genera: Gyrovirus with 9 species recognized in 2023 and the Torque group of viruses with several subgenera and many species (16). CIAV was originally classified as a Circovirus but has been reassigned to the genus Gyrovirus in the *Anelloviridae*. Table 1 summarizes the division of these viruses into the different groups. Viruses linked to chickens are identified as recognized by the International Committee on Taxonomy of Viruses (ICTV) or not (yet) recognized by the ICTV. With the exception of CIAV [renamed by the ICTV as Gyrovirus chickenanemia (16)] none of the other chicken viruses listed in Table 1 have been isolated in cell culture, which complicates experimental infections. It must be emphasized once more that *Circoviridae* and *Anelloviridae* need dividing cells for their replication thus targeting especially developing cells of the immune system and the epithelial cells in the crypts of the intestinal tract.

Avian gyrovirus 2 (AGV2, renamed by the ICTV as Gyrovirus galga 1 or GyG1) was described by Rijsewijk *et al*. in 2011 (17) from a diseased chicken in Brazil and shortly afterwards in other parts of the world (18). Thus far, GyG1 has not been linked to a specific disease. It is worrisome that several vaccines produced in different parts of the world from SPF embryos tested positive for CIAV and GyG1 (19) suggesting that GyG1 may be vertically transmitted in SPF chickens. Coinfections of individual chickens with CIAV, GyG1 and GyH1 (Gyrovirus homsa 1, previously known as Gyrovirus 3 or GyV3) has been reported causing possible recombination events (20).

GyH1 was originally isolated from children with diarrhea in different parts of the world, subsequently in stool samples from healthy children, different mammalian species and chickens (discussed in 21). Using random-PCR assays an isolate, SDAU-1, of GyH1 was constructed from a broiler flock experiencing transmissible viral proventriculitis (TVP) (22). Prior to the construction of SDAU-1, the material was found to be negative for CIAV and viruses commonly associated with TVP. Li *et al*. (21) prepared fluid containing the SDAU-1 isolate of GyH1 from a broiler kidney sample and inoculated one-day-old SPF chickens by the intra-abdominal route. Samples were collected from 29 tissues between 2-35 days post infection. Viral titers were determined by qPCR and tissue sections were analyzed for viral proteins by immunohistochemistry. Morbidity and mortality were 90 and 20% respectively. Viral proteins and high viral titers were detected in many organs including thymus, BF, and spleen indicating the potential to cause immunosuppression. In a subsequent study, it was shown that co-infection of CIAV and GyH1 synergistically enhanced the lesions caused by infection with only CIAV or GyH1 (23). A serological study found that infection with GyH1 is wide-spread in chickens in China (24). Thus far, the presence of GyH1 has not been investigated in chicken flocks in the USA. Information is not available about the relevance of GyG2 as a pathogen.

Evidence for the presence of torque viruses, the other genus in *Anelloviridae*, in chickens is limited to one publication and only based on homology of open reading frame (ORF) 2. This was detected in plasma of one of 117 chickens tested in Brazil (25). This could have been, in my opinion, a contaminating mammalian torque virus from a laboratory contamination or from a cell-cultured vaccine prepared with bovine or swine serum.

Two viruses belonging to the *Circoviridae* have been reported in chickens. Li *et al*. (26) isolated a circovirus (CCV-SDWF) from a commercial broiler flock experiencing diarrhea, 30% morbidity and 12% mortality at 21 days of age. PCR assays for gastro-enteritis associated viruses and CIAV were negative. High throughput virome sequencing yielded a circovirus. PCR assays demonstrated the presence of CCV-SDWF in 12 diseased birds but not in eight healthy chickens. The same group also identified a cyclovirus in samples obtained from commercial broilers with TVP, but the authors stated that the role of this virus in the disease was unclear (27).

METAGENOMIC METHODS TO DETECT NEW VIRUSES

Most of the viruses described in the previous section were detected using high throughput methods. In a recent review Afonso and Afonso (28) describes the use of this technology in great detail. There are two methods using next-generation sequencing (NGS): direct-targeted (tNGS) and direct-non-targeted, (ntNGS). The tNGS is used if there is a suspicion of the presence of specific pathogen(s) and is hypothesis driven. The ntNGS does not require a hypothesis and is more a general fishing expedition to find out what is present in the biome. Both techniques can be used on all types of samples including FTA card and paraffin embedded samples. Depending on the technology, short- or long-read bar-coded sequences are generated, which then go into a complicated analysis to identify sequences related to pathogens. It is expected that

artificial intelligence will facilitate the analysis of the generated sequences.

Two recent and quite different examples of metagenomics in poultry virology are an analysis of respiratory viruses using tracheal swabs collected from a broiler flock maintained at the University of Delaware (29). Samples were collected at placement and weekly samples collected afterwards until 49 days of age. Eighty-eight percent of the DNA reads (88%) were mapped to the chicken genome. During the eight-week period some viruses appeared at 4 weeks of age (*Birnaviridae* and CIAV) while coronavirus was detected in all samples. Another example is the use of metagenomics to demonstrate that MDV was present in chickens at least 1000 years ago (30). After finding MDV-specific reads, the authors were able to develop the full MDV sequence and demonstrate that the ancient strains were likely incapable of tumor formation.

CONCLUSIONS

Ideally diagnosis of poultry diseases starts with a farm visit but must always include a complete postmortem examination including the thymus in age-appropriate birds. Additional tests may be needed, which often include traditional PCR assays and increasingly real time quantitative PCR tests not only to detect the presence of a viral genome but hopefully also mRNA expression. Metagenomics will become a valuable tool in the near future to diagnose more complex and often multifactorial diseases and to find new pathogens.

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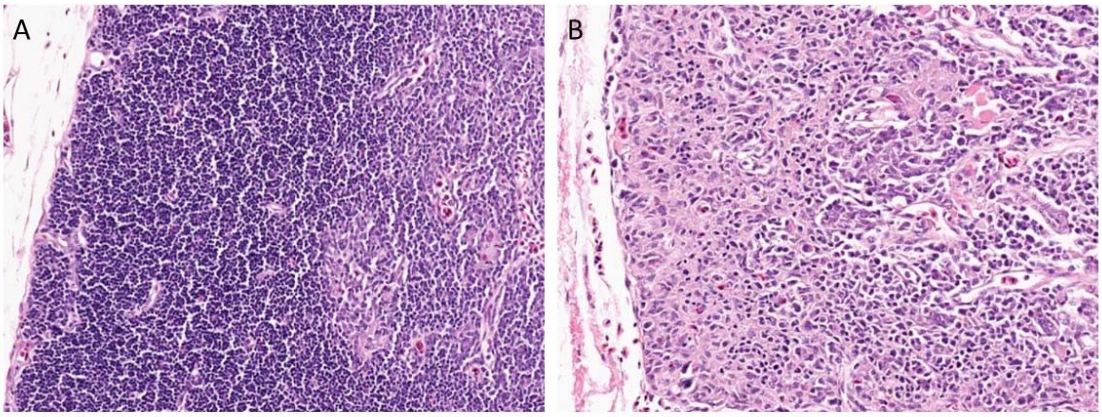
Table 1. Current classification of *Circoviridae* and *Anelloviridae* based on reports from the International Committee on Taxonomy of Viruses, (Virus Taxonomy: 2022 Release: ICTV_Master_Species_List_2022_MSL38.V3).

Virus Family	*Circoviridae*		*Anelloviridae*	
Main differences:	Ambisense genome Replication associated protein (REP)		Negative sense genome Lacks REP	
Genus	*Circovirus*	*Cyclovirus*	*Gyrovirus*	*Torquevirus*
No of genera	1	1	1	29
No of species[a]	60	88	10	145
No of species in chickens recognized by ICTV	0	0	3 (4[a])	0
No of species in chickens not recognized by ICTV	1	1	0	1

[a] Includes a species listed as Gyrovirus homsa 1 but has also been reported in chickens

Figure 1. Cortex in a normal thymus from a 14-day-old broiler chicken (A) and in a thymus from a hatch mate infected with chicken infectious anemia virus. Note the decreased cellularity in 1.B. Photos graciously provided by Dr. Oscar Fletcher.



HOW GOOD MANAGEMENT PRACTICES CAN BE IMPORTANT IN REDUCING OR ELIMINATING PAIN, STRESS, AND EUTHANASIA OF POULTRY SPECIES

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SUMMARY

Ensuring good welfare for poultry species is about giving birds a good life. David Fraser discussed this in terms of biological health, affective states and natural living. With respect to the first two components particularly, quality of bird management can ensure good health and positive affective states. Yet the importance of quality management is sometimes overlooked, and tools in the management toolbox may vary dependent on the strain of birds. This can include components of lighting programs for broilers and turkeys, stocking density for turkeys, rearing systems for pullets and others.

INTRODUCTION

The World Organization for Animal Health (2012) adopted the "10 General Principles for the Welfare of Animals in Livestock Production Systems" in 2012 (1). These general principals were adopted due to veterinary epidemiology, environmental physiology, environmental design, comparative psychology, animal handling, animal nutrition, microbiology, animal behavior and stress physiology (1). Stress in animals is a biological response to maintain their homeostasis in response to changes, while a stressor may or may not result in the alteration of homeostasis and elicit a stress response (2). Pain can also alter homeostasis of an animal due to a noxious stimuli and perceived pain. Intense stress can alter pain and may result in either increased or decreased sensitivity (2).

MATERIALS AND METHODS

The 10 General Principles have major implications in the poultry sector. Briefly the general principles include (3):

1. General selection needs to take into account animal health and welfare. *E.g.* laying hens have been selected for increased egg production, in return there is a decrease in bone calcium levels leading to osteoporosis and increased risk of injury.
2. The physical environment, as well as substrate (walking/resting surface) must be suitable for the species and breed to reduce and minimize injury and disease transmission risks. *E.g.* ulcerative pododermatitis may occur in free run poultry with inappropriate barn conditions, while hyperkeratosis can be found in in caged layers.
3. Physical environment allows for comfortable rest, movement, normal postural changes and the opportunity to perform natural behaviors animals are motivated to perform.
4. Social grouping of animals can allows for social behaviors to be performed and may (or may not) minimizes injury, disease and chronic fear.
5. Air quality, temperature and humidity in confined or open spaces need to support good animal health and not be aversive to animals. Animals should not be prevented from using their normal methods of thermoregulation when faced with extreme conditions. *E.g.* high ammonia levels causes keratoconjunctivitis, lung and tracheal damage, as well as reduced feed efficiency (25-60 ppm).
6. Access to sufficient feed and water is suited to their age and needs to maintain normal health and productivity, prevent prolonged hunger, thirst, dehydration and malnourishment. *E.g.* Inappropriate feeder space leads to competition therefore reduced intake in poultry.
7. Diseases and parasite are prevented and controlled as much as possible through good management. Birds with serious health problems should be isolated, treated quickly or humanely euthanized if treatment is not feasible or if unlikely to recover. *E.g.* good biosecurity at poultry facilities reduces the risk of contact with wild birds and rodents. This can decrease the risk of avian influenza, *Salmonella* infections etc.
8. If painful procedures cannot be avoided, the pain that results is managed to the extent that

the available methods allow. *E.g.* Beak treatment (layers, broiler breeders), using an infrared light, reduces the risk of injury, death, feather pecking, cannibalism etc.

9. Handling should foster the relationship between humans and animals, and shouldn't cause injury, pain, lasting fear or stress. *E.g.* harsh handling can depress immune function.
10. Owners and handlers have the sufficient skills and knowledge to ensure animals are kept in accordance with these principles. *E.g.* Good handling skills to reduce injury during layer depopulation.

DISCUSSION

Good quality management practices, poultry knowledge and expertise can majorly influence the health and welfare of poultry flocks. Over the years, animal ethics has become an important area of concern, with the majority of veterinarians, scientists and producers facing major ethical dilemmas on a daily basis (4). The above guidelines give a framework that encompasses poultry caregivers, handlers, owners and veterinarians.

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ASSESSING THE RISK OF MOVING DUCK HATCHING EGGS OUT OF AN HPAI CONTROL AREA

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SUMMARY

In 2022, an outbreak of highly pathogenic avian influenza (HPAI) impacted commercially-raised ducks in the United States for the first time. Animal health regulators and duck producers had to not only manage infected duck premises, but also navigate challenges related to continuity of business and outbreak containment as duck farms were caught in HPAI control areas (CAs). The risks of poultry products moving from not-known-to-be-infected (i.e., monitored) conventional poultry farms located in HPAI CAs have been extensively examined via science-based risk assessments (RAs) as part of the Secure Poultry Supply (SPS) plan; however, the duck commodity has yet to be included within the SPS plan. Thus, the risks of moving ducks, day-old ducklings, and duck hatching eggs off monitored farms are not completely understood. Here, we present the current progress and some preliminary results of the first RA that evaluates the movements of duck products (specifically duck hatching eggs) out of CAs.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) is a notifiable foreign animal disease (FAD) that is of prominent concern in the United States (US) (1). A characteristically destructive disease upon infection, HPAI spreads rapidly within flocks and results in high mortality in many gallinaceous species (2,3). As a reportable disease, HPAI causes immediate trade implications when detected and outbreaks must be promptly contained. Thus, to effectively control outbreaks of HPAI, regulatory authorities require immediate stamping out of infected flocks upon detection of the virus. Producers whose flocks become infected with HPAI suffer significant financial and emotional impacts as they rush to complete depopulation efforts. In addition, producers whose farms are not known to be infected, but are caught within the 10 km control areas (CAs) around infected premises, are immediately issued strict movement control orders. With CAs sometimes remaining active for as long as 120 days (4), such movement controls can cause immediate and, in some cases, irreparable financial, production, and welfare impacts if animals and/or animal products that are scheduled for market are unable to complete scheduled moves to their appropriate destinations.

In order to minimize the unintended consequences experienced by affected farms (i.e., not known to be infected farms located in CAs), state regulatory authorities grant producers permission to move animals or animal products from affected farms via continuity of business (COB) permits. To ensure that animals and animal products are not inadvertently spreading HPAI virus (HPAIv) as they exit or move within a CA, many states follow the Secure Poultry Supply (SPS) guidelines. The SPS plan uses science-based risk assessment (RA) to evaluate the risks of spreading HPAI associated with the movements of specific poultry products out of or within CAs. Risk assessments for each movement yield risk-based permit guidance documents that contain commodity- and product-specific pre-movement criteria to be followed by industry members. Secure Poultry Supply plan permit guidance documents have been used during the 2014-2015 (5), 2016 (6), 2017 (7), and, 2022-2024 (8) US HPAI outbreaks with success.

During the most recent—and ongoing—HPAI outbreak in the US (i.e., the outbreak that began in winter 2022), the commercial duck commodity was impacted for the first time (9). Not only did commercial meat and breeder duck farms become infected with HPAI, but commercial duck companies found their farms landing in CAs for the first time as well (Secure Duck Supply [SDS] Hatching Egg Work Group, personal communication, September 6, 2023). While not as prominent within the commercial poultry industry as the broiler, egg layer, and turkey commodities, commercial duck production is a significant commodity in its own right. Often (but not exclusively) contracting with Amish farmers (10), companies that produce ducks, duck hatching eggs, and day-old ducks include not only large, vertically integrated companies, but also smaller, independent companies and producers as well as large mail-order hatcheries that contract with or own numerous breeder farms (SDS Hatching Egg Work Group, personal

communication, September 28, 2023). Pekin ducks (*Anas platyrhynchos domesticus*) are the primary breed used in production (10,11); however, meat, eggs, and other niche products are also produced from other breeds and, in some cases, other species of ducks. Niche products include not only foie gras, but also specialty food products derived from washed and sanitized duck hatching eggs (SDS Hatching Egg Work Group, personal communication, September 28, 2023). Thus, the commercial duck commodity, while similar to other poultry commodities in some respects, is unique and diverse in others. Moreover, state and federal regulators are not as familiar with this comparatively smaller, niche poultry commodity; therefore, understanding how to move ducks and duck products out of or within HPAI CAs has proven to be challenging for animal health regulators.

Challenges involved in moving ducks and duck products were especially apparent given that, unlike other poultry commodities, including broilers, egg layers, turkeys, and upland game birds, at the time that duck farms started to become affected by the HPAI outbreak, the commercial duck commodity did not possess its own secure plan under the SPS. To serve this commodity and to help remedy challenges experienced by both regulators and producers, the National Animal Disease Preparedness and Prevention (NADPRP) program has provided funds for the initiation of the Secure Duck Supply (SDS) plan, which will provide the duck industry with commodity-specific resources and help to familiarize animal health regulators with this industry. Initial efforts of the SDS plan include conducting a single RA in which the risk of spreading HPAI associated with moving duck hatching eggs from a duck breeder farm and/or hatchery is determined. Herein, we discuss the current progress of this first RA that will inform the SDS plan.

Note that all of the results presented are considered preliminary results as of December 2023.

MATERIALS AND METHODS

To assess the risk of spreading HPAI associated with the movement of duck hatching eggs out of or within an HPAI CA, a multi-method, science-based RA was designed and is currently being conducted. The RA framework is adapted from the World Organisation of Animal Health (WOAH) Import Risk Analysis methodology (12). Specifically, the origin premises from which product is moving acts as a surrogate for the "export nation" and the destination premises acts as surrogate for the "import nation" within the WOAH risk analysis framework. Within this framework the following components are constructed including the RA scope and assumptions, industry background, hazard identification, pathogen entry assessment, pathogen exposure assessment, and overall risk determination. Given the progress of the RA as of December 2023, the methods for only the Scope and Entry Assessment will be described herein. Methods for the remaining components of the RA will be communicated in the final RA and at future conferences.

Each RA component is conducted in collaboration with the SDS Hatching Egg Work Group, a public-private partnership currently comprised of 26 members, including key commercial duck industry members and representatives from private companies and processors, state and federal animal health regulators, as well as academic, extension, and other relevant subject matter experts as needed. Serving as the critical link to the stakeholders that use the outputs of the RA, the work group (WG) collectively characterizes the scope and assumptions of RA, evaluates risk pathways, determines feasible mitigation strategies, and provides input to the risk rating for the movement(s) being evaluated. The WG is led by a risk analyst from the Secure Food Systems team (University of Minnesota, College of Veterinary Medicine) and includes the participation of relevant subject matter experts, investigators, mathematical modelers, collaborators and invited participants.

Appropriate scoping of the RA allows for feasible and timely completion of the RA, inclusion of the appropriate amount of variability, and, ultimately, control of the amount of uncertainty present within pathogen entry and exposure assessments. To determine the scope of the RA, the commodity sector is characterized with the assistance of the WG. Specifically, the products, i.e., hatching eggs and hatching egg derived products, that will be moving from an origin premises to a destination premises are identified and defined. Further, the types of origin premises as well as the types of destinations are identified and defined. Variation among product types, origin premises, destination premises, and mechanisms of product movement are captured. Production, management, and market practices and protocols are described and used to elucidate areas in which further refinement of the scope is needed.

Within SPS hatching egg RAs, the entry assessment determines the likelihood that hatching eggs, hatching egg derived products, and egg handling material will become contaminated with HPAIv. Further, the entry assessment evaluates and estimates the likelihood of contaminated products and materials introducing virus onto a destination premises (e.g., a hatchery) (13–15). To conduct the entry assessment, the biological pathways necessary for HPAIv to be introduced onto a destination premises via the movement of hatching eggs, hatching egg derived products, egg handling material, delivery vehicles, and

delivery drivers from an origin premises in a CA (e.g., a breeder farm or hatchery) are identified and qualitatively evaluated. Current egg production, handling, and shipping practices from origin premises are described by the WG. After evaluating these practices, outbreak specific preventive measures (i.e., control measures to be implemented in the event of an HPAI outbreak) are determined based on feasibility of such mitigations for commodity-specific origin premises and are evaluated for their efficacy based on the scientific literature specific to HPAI survival, virus persistence in the environment, and shedding and transmission in domestic duck species (*Anas platyrhynchos domesticus* and *Cairina moschata*).

Additionally, a disease transmission model and an active surveillance model are each built in order to characterize HPAI disease in infected ducks within a breeder house, allowing for the determination of 1) the length of latently infected and infectious periods in ducks and 2) the quantitative likelihood that eggs laid by HPAI infected ducks are contaminated with virus. Ultimately, the models can predict the number of internally contaminated hatching eggs moved from an infected but undetected duck breeder house given specified holding times after production. Briefly, mathematical models are constructed using methods as described in Malladi *et al*. 2015 (16) and are parameterized using field data collected from commercial duck producers and the available scientific literature.

Upon completion of qualitative and quantitative analyses, a descriptive likelihood rating of virus entry is determined using ratings similar to those described by Peeler *et al*. (2015) (17).

RESULTS DISCUSSION

As of December 2023, the preliminary scope and assumptions of the RA have been drafted and the entry assessment is in progress. Herein, the current progress of each section will be summarized, with the next steps outlined.

Preliminary Scope

Products covered within the risk assessment. Products included within the scope of the RA include duck hatching eggs and specialty products derived from washed and sanitized hatching eggs that are destined for human consumption. All specialty products originate from the hatchery, with some washed and sanitized hatching eggs at the hatchery being segregated away from the supply of eggs that are destined to become day-old chicks to instead become these products (SDS Hatching Egg Work Group, personal communication, September 28, 2023). Specialty products include fresh duck eggs, balut, and penoy. Definitions of specialty products are provided in Table 1.

Origin premises. Origin premises within the scope of the RA include premises from which duck hatching eggs and hatching egg-derived specialty products are moving. These premises include: 1) commercial meat duck breeder farms (i.e., company-owned or contracted multiplier, parent, grandparent, or great grandparent farms that provide duck hatching eggs for a commercial, integrated meat duck company system); and 2) company-owned or contracted farms that provide hatching eggs for a mail order hatchery company (with or without hatcheries on-site producing day-old ducklings). Specifically, such origin premises must meet the following criteria: raise domestic ducks (i.e., breeds within the species of *Anas platyrhynchos domesticus* and/or *Cairina moschata*); produce duck eggs that will be shipped to a commercial hatchery or immediately processed at a commercial hatchery building onsite; participate in the USDA-APHIS National Poultry Improvement Plan (NPIP) as stated in 9CFR145 and 9CFR147 or possess a biosecurity plan that is equivalent to the NPIP 14-point biosecurity plan; and, implement the outbreak specific mitigations as described in the SDS hatching egg RA. Additionally, unique to the duck hatching egg RA compared to hatching egg RAs of other poultry commodities, there is the overlap of origin and destination premises within the scope. As described by the WG, hatching eggs are often shipped from a centralized hatchery to other company hatcheries to fill hatching egg quotas for the region, thus origin premises also include off-site commercial hatcheries producing day old ducklings, washed and sanitized hatching eggs, and hatching egg-derived specialty products (SDS Hatching Egg Work Group, personal communication, September 28, 2023). Specifically, such origin premises must meet the following criteria: Participate in the USDA-APHIS NPIP as stated in 9CFR145 and 9CFR147 or possess a biosecurity plan that is equivalent to the NPIP 14-point biosecurity plan and implement the outbreak specific mitigations as described in the SDS hatching egg RA.

Destinations premises. Destination premises within the scope of the RA include commercial duck hatcheries, including those that are on-site with breeder duck barns as well as those that are off-site as described above. Additional, destinations for hatching eggs include depots directly accessible by customers (e.g., post-offices), and customer residences via post. Further, destinations for specialty products derived from washed and sanitized hatching eggs include distribution centers and food retailers (SDS Hatching Egg Work Group, personal communication, September 28, 2023).

Movements covered. Preliminary movements covered under the scope of the RA include those described in Table 2.

Scope next steps. The scope as described above is still preliminary and pending WG review. Further refinement of the scope may occur during the risk evaluation process (i.e., during analyses conducted as part of the entry and exposure assessments).

Entry Assessment

Within the entry assessment, the origin premises are assumed to 1) have at least one infected but undetected bird onsite prior to the movement of hatching eggs and egg handling materials off the site (in the case of premises with ducks onsite); or 2) have received contaminated but undetected eggs (in the case of premises without ducks onsite). To evaluate the likelihood of contamination, biological pathways written as specific likelihood statements were adapted from findings within previous hatching egg RAs in other poultry commodities (13–15). These likelihood statements are described below.

Likelihood of duck hatching eggs moved from an HPAI infected but undetected breeder premises being contaminated with HPAIv. Risk factors as they relate to duck hatching eggs becoming contaminated include 1) HPAI virus contamination of eggs and 2) late detection of HPAI infection in a flock. Preventative measures that take place at breeder farms as they pertain to minimizing virus contamination and as described by the WG include frequent egg pick-up in breeder flock houses, dry cleaning of eggs prior to placement in trays and/or flats in which eggs will be shipped to hatcheries, and disposal of eggs that are dirty/wet enough to impact hatchability (SDS Hatching Egg Work Group, personal communication, October 10, 2023). Outbreak specific measures including washing and sanitizing eggs at the breeder farm, fumigation of eggs at breeder farm, extended hold times of eggs prior to movement off the breeder farm and/or a combination of these measures are currently being discussed with the WG. Evaluation of current and outbreak mitigation measures to prevent contamination is currently in progress. Additionally, mortality and egg production data from HPAI-impacted duck breeder farms have been acquired, deidentified, and summarized, and disease state parameters required for the disease transmission and active surveillance models are currently being determined based on the available scientific literature.

Likelihood that duck hatching egg-handling material loaded onto a vehicle at an HPAI infected but undetected breeder premises are contaminated. Risk factors as they relate to egg handling material becoming contaminated include 1) late detection of HPAI infection in a breeder flock, 2) movement of personnel and equipment between the breeder house and egg staging areas, 3) cross contamination of egg handling material via personnel, and 4) activities involved in loading eggs on the truck. Current preventative measures that take place at breeder farms as they pertain to reducing contamination of egg handling material are currently being discussed with the WG.

Likelihood of the vehicle or driver moving duck hatching eggs from an HPAI infected but undetected breeder premises being contaminated. Risk factors as they relate to vehicles and driver becoming contaminated include 1) contamination of the egg storage room floor, 2) contamination of passage/hall ways to the egg pick-up area, and 3) the presence of a high proportion of infectious birds in an undetected breeder flock. Current preventative measures that take place at breeder farms as they pertain to reducing contamination of egg delivery vehicles and drivers are currently being discussed with the WG.

Entry assessment next steps. Qualitative analysis of biological pathways and their corresponding risk factors will be conducted using the available literature as described in the *Methods and Materials* section. Additionally, current preventative measures as well as feasible outbreak measures for mitigating the risk factors related to moving contaminated egg handling materials as vehicles and drivers will be determined in collaboration with the WG. Biological pathways will also be framed and evaluated under the context that origin premises do not have ducks onsite (i.e., centralized hatcheries). Finally, the disease transmission and active surveillance models will be constructed and validated to inform the descriptive likelihood of HPAIv entry rating along with qualitative findings from the pathway analyses.

Risk Assessment Next Steps

As work continues on the entry assessment and the scope is further refined, the hazard identification and industry background sections of the RA are concurrently being drafted to allow for qualitative evaluation of biological pathways. Upon completion of the entry assessment, the exposure assessment will be conducted, with the descriptive likelihood ratings of both sections being used to determine the overall risk of the specific product movements covered within the RA.

CONCLUSION

The current progress on the duck hatching egg RA is the first step in systematically determining the risk of moving duck-specific products out of or within an HPAI control area. The scope of the RA encompasses multiple sections of the duck hatching egg commodity sector and identifies that specialty products are additional outputs from duck hatcheries that could also be accommodated by COB permitting. The current preliminary results of the RA scope and entry assessment reveal that, given the multiple products derived from hatching eggs as well as the variation that exists among the sections within the duck hatching sector, RA findings will need to be translated into multiple permit guidances. With multiple permit guidances available, regulators and industry will be able to more appropriately and safely move duck hatching eggs and their associated specialty products out of or within HPAI Control Areas and accommodate the different types of farms that exist within the duck hatching egg sector.

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Table 1. Specialty products for human consumption derived from duck hatching eggs.

Product	**Definition**	**Reference**
Fresh Duck Eggs	Fertile, washed and sanitized duck eggs that are not incubated	SDS Hatching Egg Work Group, personal communication, September 28, 2023
Balut	Fertile, washed and sanitized duck eggs that are incubated for roughly 17 days	SDS Hatching Egg Work Group, personal communication, September 28, 2023
Penoy	Fertile, washed and sanitized duck eggs that are incubated for roughly 8 days	SDS Hatching Egg Work Group, personal communication, September 28, 2023

Table 2. Hatching egg and hatching egg-derived specialty product movements covered under the risk assessment.

Product Type	**Moving from (i.e., Origin Premises)**	**Moving to (i.e., Destination Premises)**
Hatching Eggs	Duck breeder flock	Centralized duck egg hatchery
Hatching Eggs	Centralized duck egg hatchery	Centralized duck egg hatchery (within the same company)
Hatching Eggs	Centralized duck egg hatchery	Duck egg hatchery (sold to hatchery outside of the company)
Hatching Eggs	Centralized duck egg hatchery	Depots directly accessible by consumers (e.g., post-offices)
Hatching Eggs	Centralized duck egg hatchery	Directly to consumer residences
Specialty Products	Centralized duck egg hatchery	Distribution centers
Specialty Products	Centralized duck egg hatchery	Food retailers

MODELING LOW PATHOGENICITY AVIAN INFLUENZA EVENTS TO AID IN EPIDEMIOLOGICAL INVESTIGATION AND DECISION MAKING

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INTRODUCTION

Early detection and rapid characterization of avian influenza cases in poultry is key to controlling its spread and containing any potential outbreak. Determining the time of introduction and within flock transmissibility of a pathogen guides decision making and focuses epidemiological investigations.

MATERIALS AND METHODS

Production and diagnostic testing data from flocks infected with low pathogenicity avian influenza (LPAI), subtype H6N1 were collected. The outbreak occurred from October 2020 to July 2021 and involved 59 premises across three companies in Minnesota. Diagnostic lab results and production parameters from six premises from one company were utilized in these estimations. Oropharyngeal swabs from dead and live birds were analyzed by RT-PCR and sera were tested by ELISA for AIV at the Minnesota Poultry Testing Laboratory. For each enrolled premises, one set of three pools of OP swabs (10 birds included per pool) and 10 individual serum samples were included per flock. Timing between sample sets collected varied.

In this analysis, a within-house stochastic a LPAI transmission model together with Approximate Bayesian Computation estimation approaches were used to estimate the most likely date of LPAI virus introduction and within flock transmissibility for the H6N1 LPAI virus-infected commercial turkey premises. The likelihood of getting certain sets of diagnostic results was evaluated with 10,000 combinations of time of introduction and adequate contact rates using the following equations from Bonney *et al.*:

$$l(x|\beta, t_{intro}) = \frac{\sum_{i=1}^{N_{iterations}} \prod_{j=1}^{N_{pcr}} P_i(N_{pcr,j}^{pos}|\beta, t_{intro}) \prod_{k=1}^{N_{sero}} P_i(N_{sero,k}^{pos}|\beta, t_{intro})}{N_{iterations}}$$

Where the probabilities of the diagnostic results are given by:

$$P_i\left(N_{pcr,j}^{pos}|\beta, t_{intro}\right) = \binom{N_{pcr,j}^{tests}}{N_{pcr,j}^{pos}} \left(Se_{pcr} p_{pcr,ij}\right)^{N_{pcr,j}^{pos}} \left(1 - Se_{pcr} p_{pcr,ij}\right)^{N_{pcr,j}^{tests} - N_{pcr,j}^{pos}}$$

$$P_i\left(N_{sero,k}^{pos}|\beta, t_{intro}\right) = \binom{N_{sero,k}^{tests}}{N_{sero,k}^{pos}} \left(Se_{sero} \rho_{s,ik}(\beta, t_{intro})\right)^{N_{sero,k}^{pos}} \left(1 - Se_{sero} \rho_{s,ik}(\beta, t_{intro})\right)^{N_{sero,k}^{tests} - N_{sero,k}^{pos}}$$

RESULTS

The 95% confidence intervals for time of introduction ranged from 9 to 61 days. 61 days was the upper bound of the simulation. The mode of adequate contact rate ranged from 0.3-6. 6 was the upper bound of the prior distribution and was derived from prior research. Results, which included samples that were not all positive, were more informative than those in which all samples in a set were positive at the time of collection.

CONCLUSIONS AND DISCUSSION

Our aim was to analyze changes in production parameters, which can indicate early infection in order to help producers "get in front of" an outbreak and make it more likely that the IAV will be contained rapidly and economically. Production parameter data proved to be too variable to be useful in the model. Improved timeliness and increased frequency of sample collection will improve results generated from the model. Early characterization of IAV will improve response time and decision making in companies. LPAI is reportable but not regulated in most states and thus, is up to companies to control. Results from this evaluation help illustrate the need for improved intra outbreak testing strategies and collaboration between academia and industry.

Determining the time of LPAI virus introduction into a flock is critical to outbreak investigation. Understanding time of introduction can help rule in or out routes and pathways of introduction. This, in addition to knowing the rate of spread, improves communication around needed interventions by taking "feelings" out of the equation. Timely decision making is key to control LPAI outbreaks. Estimates of time of introduction and pathogen characteristics provide a valuable tool for making these decisions.

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EXPLORING FOCAL DUODENAL NECROSIS: A PRELIMINARY STUDY INVESTIGATING DISEASE REPLICATION THROUGH CHALLENGE EXPERIMENTS

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SUMMARY

Focal duodenal necrosis (FDN), an intestinal disease, causes significant economic losses to the table egg industry. However, the etiology and pathogenesis of this disease are still unclear. This study presents results from a preliminary challenge experiment aimed at replicating the specific lesions of FDN in commercial layers. Thirty laying hens were divided into five groups. Each group were subjected to 14 days of daily oral challenge using different bacterial cocktails, which included *E. coli*, *Clostridium perfringens, Enterococcus faecalis, Gallibacterium anatis,* and *Clostridium colinum*, followed by a one-week pause. At seven-day intervals, necropsy was performed to examine for pathological changes in the duodenum. Lesions observed included mucosal hyperemia, red foci/patches, and erosions.

A notable increase in lesion scores was observed in each treatment group at each interval sampled. Histopathological analysis revealed villous tip necrosis with mucosal exudate mixed with different shaped bacteria. This pilot study offers valuable insight into replicating FDN in layers.

INTRODUCTION

Focal duodenal necrosis (FDN) is an intestinal disease of table egg layers, which is considered one of the top five concerning diseases of the table egg layer industry (1). The economic impact of FDN is associated with a decrease in egg case weight and a drop in egg production. Affected chickens may or may not have subclinical or non-specific signs such as lower body weight and pale comb (2). Although FDN was first described in 1996, the etiology of the disease has not been fully elucidated. Some studies have associated FDN with *Clostridium* spp. (3, 4). In 2016, Franca *et al*. reported an association between beta2-positive *C. perfringens* type A with FDN in egg layers in the United States (4). A challenge experiment was conducted to try to reproduce FDN using different *C. perfringens* isolates as well as duodenal homogenates obtained from FDN lesions. However, the challenge study failed to reproduce the characteristic microscopic lesions which are typically found in birds afflicted with FDN but enteritis lesions were observed (5).

To determine and further explore the etiology and pathogenesis of FDN our research group used laser capture microdissection (LCM) to excise bacteria-containing lesions, followed by 16S rRNA gene sequencing for bacterial identification (6). Analysis of the relative phylum abundance revealed differences in the duodenal microbiota and their composition between layers with FDN and healthy birds. Lesion samples were also subjected to enrichment for bacterial detection, and we identified 39/47 isolates as *E. coli*. PCR analysis for 19 *E. coli* virulence genes associated with intestinal disease including inflammatory bowel disease (IBD) found 11/39 (28.2%) isolates containing more than 10 virulence genes. This research provides insight of the correlation between *E. coli* isolates from FDN lesions and similar such isolates associated with IBD.

Other currently unpublished work from our research group involved performing DNA extraction directly from FDN fresh lesions, followed by 16S rRNA sequencing. We have also identified the presence of *Clostridium colinum* and *Gallibacterium anatis* in these samples. Additional work involving bacterial culture revealed the prevalence of*Enterococcus* in FDN fresh samples. Therefore, we have included all potential bacterial players mentioned above in this challenge study.

MATERIALS AND METHODS

Inoculum preparation and titration. The challenge strains were retrieved from frozen stocks and cultivated in specific broths suitable for each bacterium - thioglycolate broth for *E. coli*, *Clostridium perfringens*, *Enterococcus faecalis*, and *Gallibacterium anatis*, and cooked meat broth for *Clostridium colinum*. Subsequently, all broths were incubated at 37°C for 24 hours, either aerobically (for *E. coli*) or anaerobically (for the rest of the isolates). The resulting inoculum was then titrated to determine the concentration of colony-forming units (CFUs) per mL. Each bacterial culture was centrifuged

to precipitate the cells, followed by re-suspension in phosphate buffered saline (PBS). Using the design described in Table 1, the various cell suspensions were mixed in equal volumes within each experimental group. Prior to oral administration, the mixed suspensions were vigorously vortexed to ensure thorough mixing.

Experimental design. Thirty laying hens (Hy-line W-36) were obtained from a local commercial flock at 35 weeks of age and randomly divided into five groups (six layers in each group). The study involved providing water and feed to the birds *ad libitum*, with a standard lighting schedule typically employed for commercial layers. Birds were reared in cages, with three chickens per cage. All groups in this experiment received a corn– soy diet containing 10% DDGS, 5% protein meal, with 20% fine and 80% coarse limestone particles (5). Starting from the fourth day after installation, the birds within each designated group, as outlined in Table 1 below, were subjected to daily oral challenges using the bacterial cocktails formulated at a concentration of 10^8-10^9 cfu/mL. This daily oral challenge continued for 14 days, after which the challenge was paused for a week. Throughout this process, the birds underwent daily monitoring for both egg production and the manifestation of clinical signs such as lethargy or a pale comb. At seven-day intervals, three birds were selected and removed from each group. These selected birds then were euthanized and examined for any potential lesions present in the duodenum. Furthermore, the mucosal surface of the duodenum was subjected to aerobic and anaerobic culturing to detect the presence of potential pathogens.

Gross and histopathological lesion score. Duodenal samples were evaluated for the presence of gross lesions including mucosal hyperemia, red foci/patches, and mucosal erosion. Each of these lesions was scored 0 = not present, 1 = present.

For histopathological lesion scores, duodenal samples were examined and scored by a pathologist using a blinding mechanism where no indication of challenge was indicated. Intestinal samples were evaluated for the presence of lymphoplasmacytic inflammation, heterophilic inflammation, hemorrhage, necrosis of enterocytes, cystic crypts and/or crypt necrosis, and inflammatory infiltrate in the lumen. Each of these lesions was scored as 0 = no lesion, 1 = minimal; 2 = mild, 3 = moderate, and 4 = marked; the sum of lesion scores was used to determine the total microscopic lesion score per chicken.

RESULTS

No clinical signs or mortality were observed in challenged or control groups. All three gross lesions for the scoring system (mucosal hyperemia, red foci/patches, and mucosal erosion) were recorded in the challenged groups. On the other hand, in control group, mucosal hyperemia was the only lesion observed. The result of gross lesion scores are shown in Table 2. The findings indicated that group 3 (*E. coli*, *Clostridium perfringens*, and *Clostridium colinum*) had the highest lesion score (2 pts) on average. The lowest average lesion score among the challenged groups was observed for group 4 (*E. coli*, *Clostridium perfringens*, *Clostridium colinum*, and *Enterococcus faecalis, Gallibacterium anatis*) with 1pt recorded throughout the three necropsies; lesion scores increased in, groups 2 and group 4 over the course of the study while the lesion score for group 3 plateaued from second necropsy to third necropsy. Histopathological changes include mild to moderate number of lymphocytes and scant plasma cells, expansion of the lymphoid-associated tissue and lymphoid tissue hyperplasia. The lamina propria of some evaluated intestines showed mild infiltration by a number of lymphocytes and plasma cells, and some enterocytes were hypereosinophilic, with vacuolated cytoplasm and were exfoliated into the intestinal lumen. The microscopic lesions did not show characteristic FDN lesions suggesting that other predisposing factors may be involved in lesion development or other pathogens that still need to be determined to successfully reproduce the specific lesion for FDN.

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Table 1. Dosing strategies for challenged groups showing challenge strains used in cocktails.

	C. perfringens	*E. coli*	*C. colinum*	*E. faecalis*	*G. anatis*
1	+	+	-	-	-
2	+	-	+	-	-
3	+	+	+	-	-
4	+	+	+	+	+
5	Control				

Table 2. Mean lesion scores observed in duodenum on necropsy.

	Group 1	Group 2	Group 3	Group 4	Control
Necropsy 1	0.5	1	1	0	0.5
Necropsy 2	1	1.5	2.5	0.5	1
Necropsy 3	2	2	2.5	2.5	0
Mean	1.17	1.5	2	1	0.5

STUDY OF NUTRI P EFFECTS IN TURKEYS INFECTED WITH *ASCARIDIA DISSIMILIS*

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SUMMARY

The anthelmintic efficacies of Nutri P (commercial preparation of plant extracted, bioactive substances including tannins) and Quebracho (a specific plant extracted tannin) were evaluated at two different rates of dietary inclusion targeting induced infections by *Ascaridia dissimilis* in turkeys. Additionally, efficacies were assessed when the plant extract dietary treatments were coupled with routine Safeguard (fenbendazole) treatment in the water. The induced infections were light, with individual control bird (N=10) infections totaling 40 to 212 nematodes with a mean of 91.5, and with all parasitic, developmental stages present at necropsy (L2, L3, L4, L5 and mature adult). No treatment regime resulted in overall efficacies set by the World Association for the Advancement of Veterinary Parasitology (WAAVP) as "efficacious" ($\geq$ 95%). For groups receiving extracts alone (Nutri P or Quebracho), total nematode levels were reduced overall by 39.1% from control levels. For those groups receiving the extracts plus fenbendazole, an overall reduction of 55.7% from control bird levels was realized for the total worm burdens. Adult, as opposed to larval ascarid level were most effectively reduced in this study. No untoward effects of treatment were seen in this study, with all treatment rations readily consumed. Bird weight gains, feed intakes and feed efficiencies did not vary appreciably between treatment groups.

This study was conducted according to WAAVP guidelines and a study-specific, University of Arkansas IACUC protocol.

INTRODUCTION

Helminth parasite burdens are commonplace in poultry production; a situation that has progressively become worse over recent years. This rise is due to many factors, including the move from caged housing to cage-free, the increased popularity of "organic" food and helminth innate or developed resistance to a very limited number of available, registered anthelminthics. The need for effective means of parasite control is definite; a need that is addressed in this research. *Ascaridia dissimilis* is the sole, omnipresent helminth parasite of commercial turkeys (1), although short-term *Heterakis gallinarum* infections are possible as well. In this research, two preparations of plant extracts (Nutri P® SilvaFeed and Quebracho) were fed in the ration at two different rates of inclusion and evaluated for anthelmintic effect against induced, *A. dissimilis* infections. Anthelmintic efficacies of the above treatments were also determined when the treatments were combined with bird treatment with fenbendazole (Safe-Guard® Merck), the most used turkey anthelmintic.

MATERIALS AND METHODS

Gathering the infective larvae. Turkey intestinal tracts were obtained from a local processing plant and adult female *A. dissimilis* were isolated and their uteri extracted and processed for egg collection. Anticipating a 30% rate of successful larval development (+/-), eggs were collected until a sufficient number of eggs were collected for the study. At that point, they were cultured in the lab (room temperature) until the infective stages were obtained in the eggs. The fully larvated eggs were then refrigerated in aeration flasks until they were used for infection inductions via the feed.

Timing of infections and bird management/treatments. After the desired number of infective larvae were obtained, day old turkey poults were obtained and reared for approximately two weeks. The birds (12 per pen) were then placed into their experimental pens (N=10) and allowed to acclimate for approximately one week. At the time of placement, the 8 pens of birds designated to get Nutri-P or Quebracho at high or low levels were started on their specified rations.

After approximately ten days of the feeding the pen-designated rations (which continued for the remainder of the study), the birds were dosed (in the feed) with larvated, ascarid eggs at the rate of approximately 575 eggs per bird per day for five contiguous days.

Three weeks after the final infection doses were given, fenbendazole was given to the birds designated to receive the anthelmintic at the customary rate of 1 mg per kg BW per day for five contiguous days. The dosage of fenbendazole given each day was calculated from the anticipated weight of the birds in five days after the start of treatment; this, to ensure that the target dosage was exceeded/delivered each day during the treatment period. Two birds from each of the ten pens were selected at random and necropsied for worm counts just before fenbendazole treatments were started. Fenbendazole was given on a per pen basis in the one pen plasson waterer each day; this, to simulate actual commercial conditions for treatment. Each day of treatment, the medicated water was observed to be entirely consumed before the normal water supply was turned back on.

The treatment groups are detailed in Table 1.

The incremental consumption of ration, as well as bird weights, were obtained on a per pen basis for the duration of the study.

Approximately one week after the final fenbendazole treatment, all remaining 10 birds per pen were necropsied for worm collections on one day.

The treatments. Nutri P (SilvaFeed) a blend of plant extracts (minimum of 70% polyphenols), at two and four pounds per ton of feed, resulting in 0.10% and 0.20% rates of ration inclusion (low and high, respectively).

Quebracho, a pure, plant extracted tannin, at two and four pounds per ton of feed, resulting in 0.10% and 0.20% rates of ration inclusion (low and high, respectively).

Fenbendazole, 10% suspension (Safe-Guard Merck, Lot # 263A01, exp. date of 5/24) at 1 mg/kg bodyweight, per day, for five days as delivered in plasson waterers with measured amounts of drinking water. Pen dosage was based on the anticipated bird weight at the end of treatment.

Parasite isolation and quantification. After bird euthanasia, the small intestine was removed from each bird, opened lengthwise, and the contents collected and sieved for mature ascarid removal and counting. The contents, sieve residue and intestinal tract were then combined and refrigerated overnight. In the morning, the intestinal mucosa was removed by drawing intestinal tract through clenched fist, and the rinse/mucosal slurry was added to the previously collected contents. The combined rinse and contents were then mixed thoroughly, and a 10% aliquot retained for subsequent sieving and stereo-microscopic viewing of the residues for identification and counting of all *A. dissimilis* stages.

Miscellaneous. All procedures followed in this study were in accordance with the most recent World Association for the Advancement of Veterinary Parasitology guidelines (2) and a study specific IACUC protocol (Ag-IACUC # 23054).

Worm count, analysis of variance was performed for multiple mean comparisons with the Tukey posthoc test. The analysis was performed with both non-transformed and transformed [$\log_{10}(X+1)$] data with significance set at the level of $P \leq .05$.

RESULTS

Treatment group, mean bird weights at the end of the study, as well as feed efficiencies on a treatment groups basis were not seen to vary appreciably between groups, with treatment group mean bird weights ranging from 3.0 to 3.2 Kg, and feed efficiencies ranging from 1.7 to 1.9 (intake to gain).

Mean, stage specific worm counts on a treatment group basis are presented in Table 2. No ascarids were found in the uninfected control group, indicating that there were no unintended environmental or induced challenges in the study. In groups 9-10, light populations of all parasitic *A. dissimilis* developmental stages were quantified. Mean total worm burdens were higher in group 2 than in group 10, with the other infected groups intermediate in magnitude ($P \leq .05$). Populations of larval stages (L2, L3 and L4) were generally equivalent between treatment groups, except for treatment group 10 burdens, which were appreciably lower than what was seen in the other groups. Adult worm (L5 and mature adults) infections were significantly higher in the infected, control group than in any of the infected, treated groups ($P \leq .05$).

DISCUSSION

Relatively light *A. dissimilis* infections were developed in this study; infections that did not alter production parameters (feed intake, weight gains and feed efficiencies), but which could be used to assess treatment efficacies. Larval worm burdens were generally not impacted by treatment with plant extracts (Nuti P or quebracho) with or without additional bird treatment with fenbendazole. Mature adult worm burdens however were significantly reduced by all plant extract treatments, with the addition of fenbendazole treatment slightly increasing the rate of mature worm removal. Birds receiving plant extracts alone (groups 2-6) experienced an overall reduction of 84% in mature ascarid populations relative to the control group (group 2). Birds receiving extracts plus fenbendazole (groups 7-10) experienced a corresponding overall reduction from control levels of 93%, only an 11% improvement from extract-alone reduction rates. Reduction in mature worm burdens has the additional benefit of immediately reducing the

level of parasite eggs added to the environment which in turn results in lowered parasite challenge to the current and future populations of birds. Total burdens were also significantly lowered by treatment, an anthelmintic effect that was also primarily the result of plant extract treatment as opposed to bird treatment with fenbendazole. No consistent correlation was seen between the dietary level of either plant extract and any of the resultant worm burdens, indicating that the levels of Nuti P and quebracho used in the study were above the realistic, commercial threshold needed for these products to achieve anthelmintic effectiveness.

This study showed that Nutri P and quebracho inclusion in the feed clearly reduced *A. dissimilis* burdens in turkeys; worm reductions that were marginally enhanced by co-treatment with fenbendazole. For a product to be deemed an "anthelmintic" by the WAAVP, it must have an efficacy of ≥95%; a level of effectiveness that was seen for some treatment groups for adult ascarid burdens (L5 and/or mature adults), but not for larval or total worm burdens. The mode of action for benzimidazoles has been clearly established and involves the ability of parasites to complete microtubule production and utilization (3). The mode of action for tannins/plant extracts to cause helminth removal is currently unclear, and may involve a combination of direct as well as indirect activities, i.e. altering gut motility (4), changing the gut microbiome (5), enhancing the integrity of the mucosal epithelium (6) and binding to essential parasite proteins (7,8). Regardless the mode of action of tannins and other plant extracts, any aid in the control of parasites would be welcome, as anthelmintic resistance is extremely commonplace and new anthelmintics are apparently not forthcoming in the short term.

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Table 1. Treatment groups (one pen per treatment).

Group	Nutri P	Quebracho	Parasite challenge	Fenbendazole
1.	No	No	No	No
2.	No	No	Yes	No
3.	High	No	Yes	No
4.	Low	No	Yes	No
5.	No	High	Yes	No
6.	No	Low	Yes	No
7.	High	No	Yes	Yes
8.	Low	No	Yes	Yes
9.	No	High	Yes	Yes
10.	No	Low	Yes	Yes

Table 2. Treatment group means for *A. dissimilis* per developmental stage at necropsy.

Treatment Group	L2	L3	L4	L5	mature adult	total
1	0	0	0	0	0	0
2	3	38	18	22[a]	11[a]	92[a]
3	2	23	22	3[b]	2[b]	52[a,b]
4	3	33	30	3[b]	3[b]	72[a,b]
5	0	12	42	2[b]	1[b]	57[a,b]
6	2	18	21	0[b]	2[b]	42[a,b]
7	0	26	29	1[b]	1[b]	57[a,b]
8	0	26	17	0[b]	1[b]	44[a,b]
9	2	26	16	3[b]	2[b]	49[a,b]
10	0	11	8	6[b]	1[b]	26[b]

[a,b] means in the same column with unlike superscripts are significantly different ($P<.05$) with analysis of variance using transformed data [$\log_{10}(X+1)$]