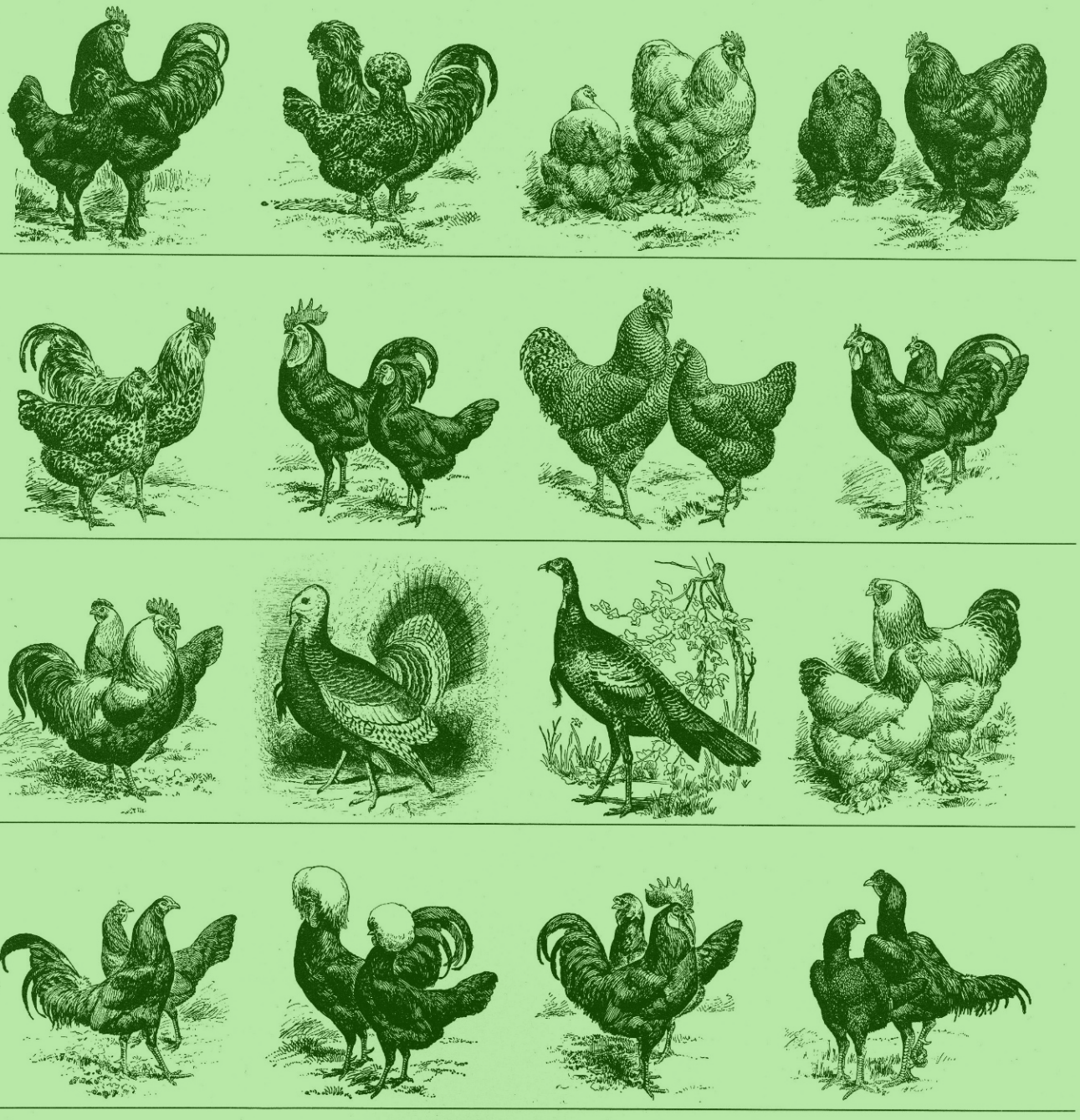
PROCEEDINGS OF THE SEVENTIETH WESTERN POULTRY DISEASE CONFERENCE

March 15 – 16, 2021 Virtual Conference

WESTERN POULTRY
DISEASE CONFERENCE



PROCEEDINGS OF THE SEVENTIETH WESTERN POULTRY DISEASE CONFERENCE

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70th WESTERN POULTRY DISEASE CONFERENCE DEDICATION

WALTER FRANKLIN HUGHES (1922-2020)

Walt Hughes was born January 27, 1922 in Santa Barbara, California. He worked hard on the family farm during the Great Depression to help the family survive. Walt later volunteered for the Army Air Force Pilot Training and subsequently flew B-24 heavy bombers deep into Nazi Germany. Although wounded once, Lt. Hughes incredibly flew the required 35 missions in the 93rd Bombardment Group of the Eighth Air Force and always brought his bomber safely back home. At March Field, Walt met his future wife, a wonderful WAAC named Violet Sasso. After the war they were married and began attending the University of California at Davis. Walt graduated with a bachelor's degree in animal husbandry and agronomy and enough credits for a third degree in chemistry.

Walt was a member of the first graduating class of the UCD School of Veterinary Medicine in 1952. While at UCD, Walt worked as a technician under the tutelage of Drs. Ray Bankowski, Henry Adler, Dick Yamamoto, and Don Zander. After graduation, Dr. Hughes first worked at a general practice in Lancaster for a while, but later moved to Petaluma to work in poultry. In 1955 Walt assumed the directorship of the veterinary laboratory at Kimber Farms. At Kimber, Walt became well-known for his scientific achievements in poultry medicine and vaccination. In 1971 he developed a California-state-licensed vaccine for MD lymphomas that was subsequently used world-wide. He also originated improved vaccination and treatment techniques for Newcastle disease control.

After a very successful and noteworthy career at Kimber, he joined Olson Farms in 1971 and was appointed Vice President of Operations at Fairview Farms (egg production division of Olson Farms).

Although a very prominent and well-known poultry scientist, Dr. Hughes never forgot his veterinary roots. Taking care of neighborhood animals and caring for their owners was very important to him. Walt was active in community affairs as well. He was a member of the Notary Club in Niles, CA and a member of the Niles Elementary School Board. He was instrumental in creating the Fremont Unified School District, which consolidated five elementary districts and the regional high schools into one city school district.

An avid gardener, Walt once accidentally developed a unique strain of zucchini from his compost pile. Is was apparently delicious but also very prolific.

Dr. Hughes regularly attended and participated in the Western Poultry Disease Conference, and received the Special Recognition Award at the 44th WPDC in 1995. It is with deep appreciation and utmost respect that we dedicate the 70th WPDC to his memory.

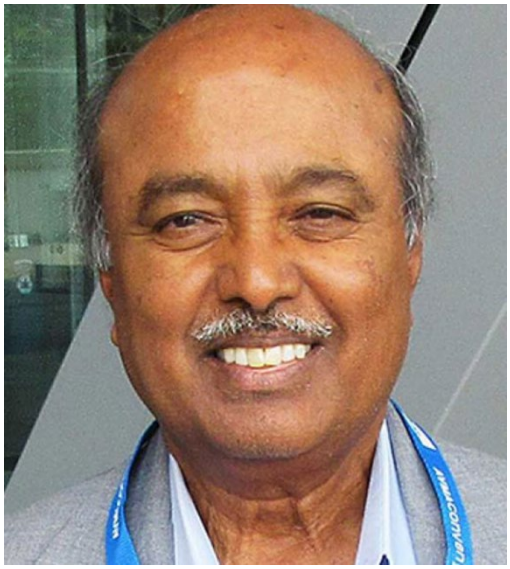
Violet and Walter while at UC Davis working on veterinary degree.

Dr. Hughes at Kimber Farms.

Walt said the secret to longevity was diet, community, and exercise; tai chi gave him both the community and the exercise. He credited it to keeping him balanced and agile. He was still climbing and pruning trees well into his 90s.

70th WPDC SPECIAL RECOGNITION AWARD

H. L. SHIVAPRASAD



Dr. Shivaprasad, known most commonly as "Prasad," obtained his BVSc from Bangalore Veterinary College, India in 1969 and his MVSc in Poultry Genetics from Uttar Pradesh Agricultural University, Pantanagar, India in 1971. He then served as a faculty member at the University of Agricultural Sciences in Bangalore, India for a short period before moving to the United States in 1973 to pursue his MS and PhD in Physiology and Genetics at The Ohio State University from 1973 to 1977. He then completed an MS and residency training in Veterinary Pathology from Purdue University in 1980. He was a faculty member in Veterinary Pathology at the University of Illinois for five years and an avian pathologist at Cornell University for four years prior to moving to California in 1989. Dr Prasad has since served as a faculty and avian pathologist at the California Animal Health and Food Safety Laboratory System (CAHFS) Diagnostic System, University of California, Davis. Dr. Prasad is board certified by the American College of Poultry Veterinarians (ACPV). In 2019, he was elected as an honorary member of the American College of Veterinary Pathology based on his extraordinary contribution to the advancement of veterinary pathology.

Dr. Prasad has been involved in avian diagnostics, teaching, and research for over 40 years. His research interests include the identification of novel disease etiologies and the pathogenesis of infectious and noninfectious diseases of poultry and other avian species. He has published over 185 manuscripts in journals, contributed to 44 book chapters, and has presented more than 450 papers in national and international conferences. He is a member of many professional organizations including the AAAP, ACPV, AVMA, AAVLD, AAV, WPDC, and the C.L. Davis and S. W. Thompson DVM Foundation for the advancement of veterinary and comparative pathology. He has served on the editorial board of *Avian Diseases* and *Journal of Avian Medicine and Surgery* and was an associate editor of the *Journal of Veterinary Diagnostic Investigation* for five years and *Avian Pathology* for two years. He is a co-editor of the *Avian Disease Manual* published by the AAAP and an associate editor of the *Manual of Poultry Diseases* published by the Association française pour l'avancement des sciences, Paris, France. He has served on numerous committees in many organizations and has been the Chairman of the Histopathology and Case report interest group in AAAP for more than 16 years. He has discovered numerous novel infectious agents, some of which are Hepatitis E virus in chickens, Muscovy duck parvovirus in ducks in the USA, a novel picornavirus in turkeys associated with turkey viral hepatitis, and most notably, avian bornavirus associated with proventricular dilatation disease in psittacines, and non-infectious diseases and conditions in various avian species. Dr. Prasad's discoveries of the etiology and pathogenesis of proventricular dilatation disease have resulted in 15 published manuscripts.

Some of the important awards he has won include, senior author of the best paper published in the *Journal of Veterinary Pathology* in 2011. The paper was recognized for its scholarship, significance and illustrations. He was also the recipient of the Lasher-Bottorff Award in 2015 awarded by the AAAP in his recognition as an eminent avian

pathologist and for his contribution to the poultry health programs in North America. The 16th 'Hafez' International Conference, held May 2016 in Berlin, Germany on turkey diseases, was dedicated to him for his outstanding contributions to the study of the pathogenesis, diagnosis, and control of turkey diseases. In 2017, he was inducted into the AAAP Hall of Honor, the highest award bestowed by AAAP in recognition of distinguished contributions to poultry health. In 2018, the Pacific Egg and Poultry Association recognized him as the Scientist of the Year. Dr. Prasad is passionate about avian diagnostics and teaching, loves sharing his knowledge with others, and has a voracious appetite for learning. He has personally trained more than 100 DVM and graduate students, post docs, residents, faculty, and researchers from countries around the globe. He has traveled to more than 30 countries on invitation, primarily for teaching in avian diagnostics and pathology.

He has been married to his wonderful wife, Kathy for more than 43 years and they have three lovely children, Sheela, Anand, and Arun. He enjoys playing golf, national and international travels, and photography. Dr. Prasad's long list of contributions to the advancement of avian pathology, his dedication to teaching, tireless mentorship to students from all over the world, and significant publication record over these 40 years are inspiring. He is a role model and mentor to many professionals in the field of avian pathology and diagnostics around the world.

It is a pleasure and privilege to present Dr. H. L. Shivaprasad with the 70th Western Poultry Disease Conference Special Recognition Award.

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SPECIAL ACKNOWLEDGEMENTS

The 70th Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and to help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. Many companies and organizations have once again given substantial financial support, including some that also send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

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

Dr. Lynn Bagley, Program Chair of the 70th WPDC, would like to thank the WPDC Executive Committee, especially Dr. Rodrigo Gallardo and Dr. David Frame, for their help and assistance. Dr. Bagley would like to thank the invited speakers who willingly gave of their time and knowledge. He also wishes to thank all student volunteers and moderators for their willingness to provide assistance during the conference. On behalf of the WDPC, Dr. Bagley also extends his sincere gratitude to all speakers who have contributed to this year's conference.

Many have provided special services that contribute to the continued success of this conference. For this year's meeting, the WPDC has contracted ACPV to put together a virtual meeting. We would like to thank the Poultry Medicine lab members at UC Davis, WPDC executive committee, and the staff of the Department of Population Health and Reproduction at the UC Davis School of Veterinary Medicine for administrative support to our conference. We particularly thank Bob and Janece Bevans-Kerr, Channah Pool, and all of the AAAP staff for their continual help.

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

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

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70th WPDC PROCEEDINGS

Please note that the proceedings of the 70th 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 70th WPDC are available in electronic format only. They can be downloaded from the American College of Poultry Veterinarians website (www.acpv.info).

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MINUTES OF THE 69TH WPDC ANNUAL BUSINESS MEETING

Because of the COVID-19 pandemic and the subsequent cancellation of the 69th in-person WPDC, an annual business meeting was not held.

FUTURE MEETINGS

Future WPDC meeting dates and locations:

2021: 70th WPDC and ACPV-sponsored workshop, to be held virtually March 15 and 16, 2021
2022: 71st WPDC and ACPV-sponsored workshop, Vancouver, BC April 9 to April 13, 2022
2023: 72nd WPDC and ACPV-sponsored workshop, Sacramento, CA

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ANTIMICROBIAL USE STEWARDSHIP IN THE CANADIAN POULTRY SECTOR: THE USE OF MULTIPLE ANTIMICROBIAL USE ANTIMICROBIAL RESISTANCE, FLOCK HEALTH AND PRODUCTION INDICES TO MEASURE ITS IMPACT

A. Agunos[A], S. Gow[B], D. Léger[A], A. Deckert[A], and R. Reid-Smith[A]

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SUMMARY

The poultry industry in Canada has implemented their antimicrobial use (AMU) reduction strategy, a phased approach for the reduction of antimicrobials from 2014 to 2020 (1). The poster presentation will aim to describe the most recent results from the CIPARS and FoodNet Canada broiler chicken and turkey farm-level AMU and antimicrobial resistance (AMR) surveillance using a sentinel-based data and sample collection approach. In 2019, our analysis indicated that the progressive elimination of certain classes of antimicrobials (preventive uses) in both species changed the diversity and the relative proportion of antimicrobial classes used while it decreased the prevalence of resistance to antimicrobials deemed of highest priority to human medicine (3rd generation cephalosporins and fluoroquinolones) and accounting for possible co-selection (multidrug resistance). This presentation highlights the value of using multiple AMU, AMR, and flock health indices in monitoring the impact of an AMU reduction strategy.

MATERIALS AND METHODS

Farm-level AMU data and samples for bacterial isolation and AMR testing were collected from a network of 16 veterinary practices and their producers. Detailed AMU data, vaccinations, biosecurity and flock health were collected using a questionnaire administered by the veterinarian/staff to the producer. For bacterial recovery and antimicrobial susceptibility testing, 4 pooled fecal samples were collected from the 4 quadrants of the barn. Detailed methods are described elsewhere (2).

RESULTS AND DISCUSSION

Data on AMU and AMR in enteric bacteria, collected from 2013 to 2019 from broiler chickens (n = 947 flocks) and from turkeys (n = 427) were used.

AMU summary. Between 2018 and 2019, the milligrams per population correction unit (mg/PCU) increased in both broiler chickens (124 to 143 mg/PCU, 15% change) and turkeys (53 to 84 mg/PCU, 57% change). When adjusted for defined daily doses for animals using Canadian standards (DDDvet), a decrease in the total nDDDvetCA per 1,000 animal-days at risk was observed (broilers: 492 to 454, -8% change; turkeys: 107 to 95, -11% change). The relative proportion of the antimicrobial classes used in 2019 in broiler chickens and turkeys are shown in Figure 1 and the proportion of medically important antimicrobials relative to coccidiostats are shown in Figure 2. The reason for AMU was largely for prevention (broilers: 83%; turkeys: 78%). Vast majority of the antimicrobials in both species were administered via feed for the prevention of necrotic enteritis.

AMR summary. Between 2018 and 2019, in broiler chickens, resistance to 3 or more antimicrobial classes (≥ 3 multiclassR) decreased by 4% among *Salmonella* and slightly increased among *E. coli* (1%) and *Campylobacter* (1%). Resistance to ceftriaxone decreased by 5% among *Salmonella* and remained stable among *E. coli;* these findings are reflective of the cessation of use of ceftiofur at the hatcheries (since May 2014). However, ciprofloxacin resistant *Campylobacter* increased by 12% despite no reported use.

During the same timeframe, in turkeys, resistance to three or more antimicrobial classes (≥ 3 multiclassR) decreased by 26% among *Salmonella*, increased among *E. coli* by 8%, and remained relatively stable for *Campylobacter*. Resistance to ceftriaxone increased slightly among *Salmonella* (2%) and *E. coli* (1%). Similar to broilers, ciprofloxacin resistant *Campylobacter* increased but of higher magnitude (30%) but with limited number of flocks treated with enrofloxacin, a veterinary fluoro-quinolone (two flocks in 2019, one flock each in 2018 and 2017).

Flock health status. In broiler chickens, the reported diagnoses of bacterial diseases including

lesions typical of colibacillosis and protozoal diseases (coccidiosis) increased. However, the average flock mortality remained relatively stable (4.1 to 4.2%). similarly, in turkeys the reported diagnoses of the same diseases diagnosed in broilers increased but of relatively lower magnitude for coccidiosis. The mortality decreased from 6.9% to 6%.

CONCLUSION

Our data indicated that the poultry industry strategy eliminating the use of certain antimicrobials for preventive use is working without any negative implications to flock health. It highlights the need for ongoing surveillance to continuously improve best management in response to the national and global call to reduce AMU and AMR.

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1. Chicken Farmers of Canada. AMU strategy, a prescription for change. 2018. Available from: https://www.chickenfarmers.ca/resources/amu-strategy-a-prescription-for-change/

2. PHAC. Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2018: Design and Methods. 2020. Available from: https://www.canada.ca/en/public-health/services/surveillance/canadian-integrated-program-antimicrobial-resistance-surveillance-cipars/cipars-reports/2018-annual-report-design-methods.html

Figure 1. Relative proportion of antimicrobial classes used in broiler chickens and turkeys in 2019

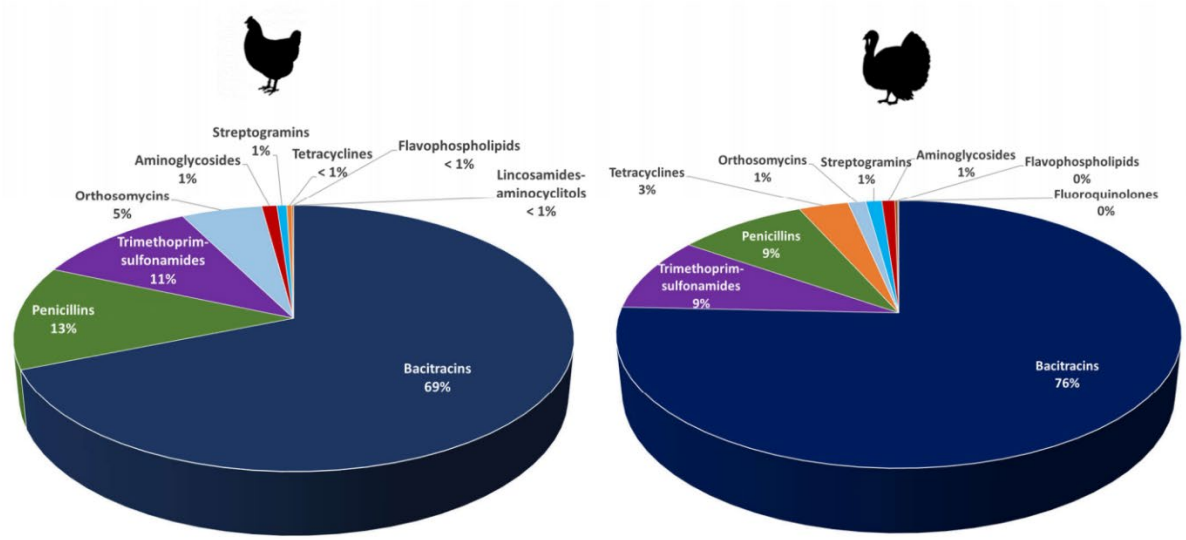
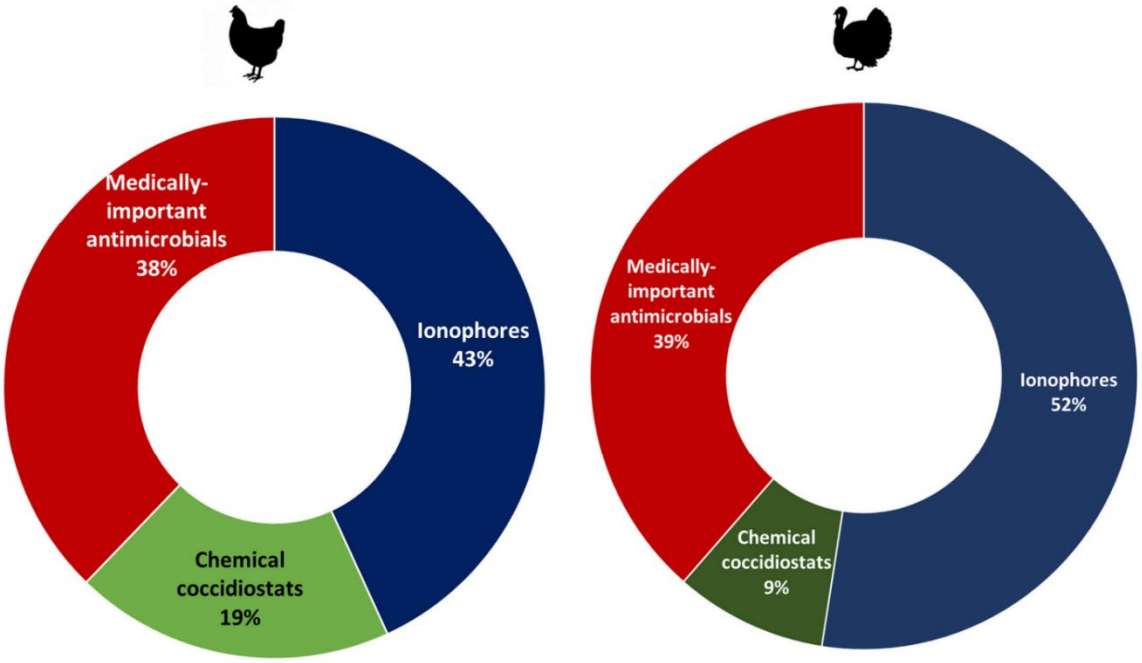


Figure 2. Relative proportion of medically important antimicrobials and coccidiostats



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

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SUMMARY

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

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

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

INTRODUCTION

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

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

MATERIAL AND METHODS

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

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

RESULTS

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

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

The average airsacculitis % in five flocks processed between January 2018 and November 2018 was 0.056%. With the implementation of GA type vaccine at this farm, average airsacculitis in the following 5 flocks processed between January 2019 and October 2019 was reduced to 0.016%. When looking at the most challenging time of the year for respiratory disease, the difference was more dramatic. There was a 4.4-fold decrease in airsacculitis condemnations when comparing flocks raised at this farm during winter 2017-2018 and winter 2018-2019 (0.11% before GA type vaccination vs. 0.025% after GA type vaccination).

DISCUSSION

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

ACKNOWLEDGMENTS

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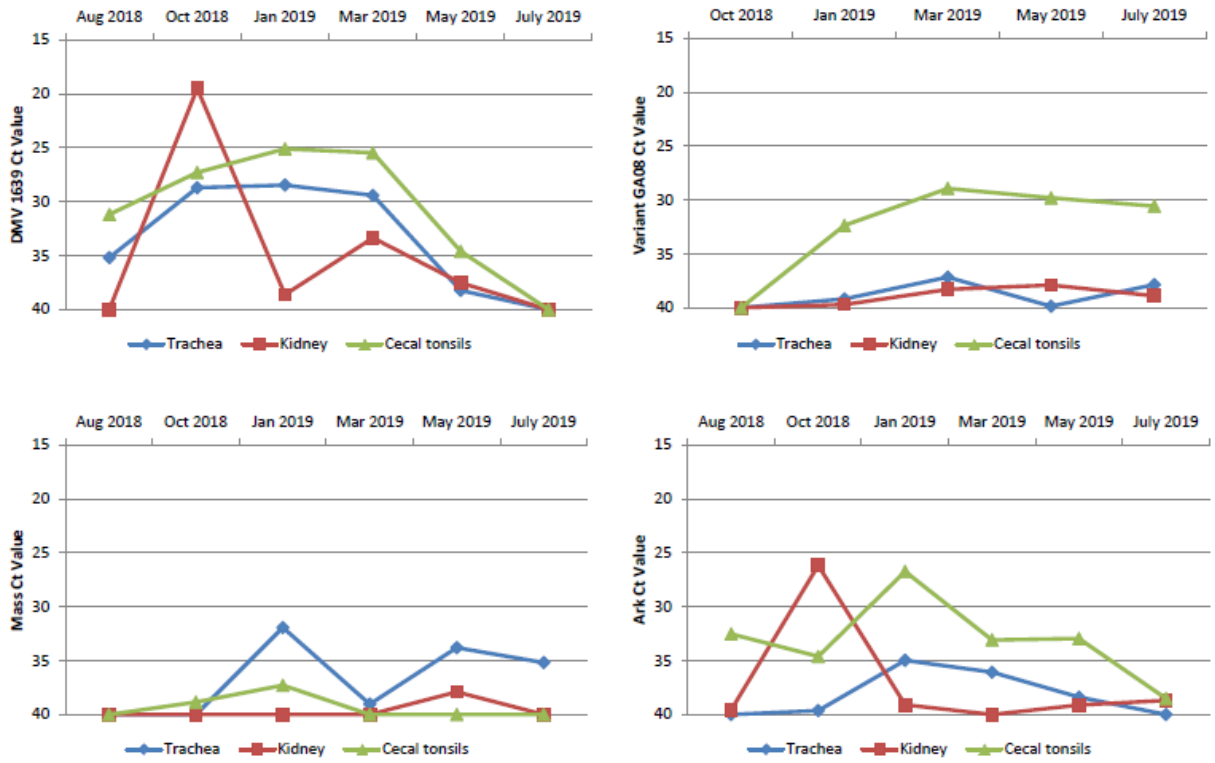
Table 1. IBV Vaccination program by flock

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

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

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

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



PROTECTION BY A DUAL RECOMBINANT HVT-ND-IBD VACCINE AGAINST THE VARIANT DELAWARE E AND AL-2 STRAINS OF INFECTIOUS BURSAL DISEASE

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SUMMARY

The objective of this study was to evaluate the level of protection provided by a recombinant HVT-ND-IBD vaccine (Innovax ND-IBD®) against two of the most prevalent variant IBD strains in the U.S., the Delaware E and AL-2 strains. The HVT-ND-IBD vaccine was administered subcutaneously to one-day old SPF chickens with challenge performed at 14 and 18 days of age. At seven days post-challenge, histological evaluation of bursa tissues was performed and bursal to body weight ratios were calculated. Bursal atrophy caused by the challenge viruses was observed, with birds in the non-vaccinated/challenged groups exhibiting the most severe bursal atrophy. In contrast, birds in the groups vaccinated with the HVT-ND-IBD vaccine showed significantly milder bursal atrophy than the non-vaccinated/challenged group.

Protection against challenge was determined using a reference bursa to body weight ratio, calculated by subtracting two standard deviations from the mean bursa to body weight ratio of the non-vaccinated/non-challenged group. Based on this criterion, the HVT-ND-IBD vaccine provided protection against bursal atrophy in birds against early challenge at 14 and 18 days of age with the Delaware E and AL-2 viruses.

INTRODUCTION

Infectious bursal disease (IBD), also known as Gumboro disease, is a worldwide highly contagious disease in young chickens. IBD presents an important threat to the commercial poultry industry due to its devastating economic impact associated with immunosuppression, increased mortality, increased feed conversion, augmented susceptibility to other pathogens and reduced efficacy of vaccination programs.

IBD prevention and control are based on biosecurity and implementation of vaccination programs designed according to the type and severity of field challenge. Historically, IBD immunization programs have relied on live attenuated and inactivated vaccines. However, recombinant turkey herpesvirus vectored vaccines (rHVT) carrying the VP2 gene have become an important tool to control IBD in broilers, breeders and commercial layers. Recently, dual rHVT carrying the VP2 gene of IBD virus and the Fusion gene of Newcastle virus (HVT-ND-IBD) have also become commercially available. Advantages associated with the use of rHVT vaccines include the absence of post-vaccinal reactions (bursa atrophy), long lasting protection and improved overall performance.

In this study, we evaluated the level of protection provided by a dual-recombinant HVT-ND-IBD vaccine against two of the most prevalent field IBD variant strains isolated in the U.S., the Delaware E and AL-2 strains.

MATERIALS AND METHODS

Birds. SPF embryos obtained from Charles River SPAFAS were incubated at the Poultry Diagnostic and Research Center (PDRC), University of Georgia. At hatch, only healthy chickens were selected for the study. Two-hundred one-day-old SPF chickens were randomly selected and equally divided in the following treatment groups:

a. HVT-ND-IBD vaccinated, Delaware E challenged
b. HVT-ND-IBD vaccinated, AL-2 challenged
c. Non-vaccinated, Delaware E challenged
d. Non-vaccinated, AL-2 challenged
e. Non-vaccinated, Non-challenged

Housing, diet and water. Birds in each treatment group were further divided in two groups of 20 chicks per group and housed in negative pressure isolation units with unrestricted access to feed and water. Birds were fed unmedicated Southern States All Grain Start-N-Grow diet and were observed daily until the end of the study.

Vaccine. A recombinant HVT-ND-IBD vaccine carrying the VP2 gene of the IBD virus and the Fusion gene of Newcastle disease virus (Innovax ND-IBD) was used in this study. One ampule of 4,000 doses of Innovax ND-IBD was removed from liquid nitrogen, thawed at 80˚F for approximately 90 seconds and mixed with 800 mL of Marek's diluent at room

temperature following the recommendations of Merck Animal Health. One mL of sterile blue dye was added to the diluent to facilitate the visualization of the vaccine post-administration. A dose of 0.2 mL was subcutaneously injected to each bird by the same person, followed by verification of the presence of blue dye under the skin of the neck. Detection of the recombinant HVT-ND IBD virus in spleen and feather pulp samples from vaccinated birds was performed using Next Generation Sequencing (NGS) technology (Viral Flex Seq®).

Challenge viruses. The challenge viruses used in this study were the 124024 (AL2-like) and the Delaware E variant IBD isolates, obtained from field submissions to the PDRC. The viruses were expanded in three-week-old SPF chickens prior to the start of the study, bursae collected, homogenized and titrated in chicken embryos. The viruses were diluted in tryptose phosphate broth (TPB) to obtain a target dose of $10^{3.0}EID_{50}$/dose. Each bird in the AL2 and Delaware E challenged groups received 0.05 mL by the intraocular route at either 14 or 18 days of age.

Samples collection. At 14 and 18 days of age (right before challenge), five birds from each group were euthanized and bursae collected for histopathological evaluation, with lesion scoring performed only in bursas obtained at 18 days. Bursal lesions were scored from 1 to 4 based on their degree of atrophy, as follows: 1 = normal to 10% bursal atrophy; 2 = 10% to 30% bursal atrophy; 3 = 30% to 70% bursal atrophy and 4 = more than 70% bursal atrophy.

At seven days post-challenge (21 and 25 days of age) birds in each treatment group were humanely euthanized, necropsied and weighted. Axillary feather pulps and spleens were imprinted in FTA cards for the detection of the HVT-ND-IBD vaccine and sequencing of specific genes using Next Generation Sequencing technology (Viral Flex Seq). Bursas were collected, weighted to determine the bursa to body weight ratio and half of the tissues were immersed in 10% buffered formalin for histopathological evaluation and scoring.

Evaluation of protection. Protection against challenge viruses was determined based on bursa to body weight ratios (Bu:Bw) obtained seven days post-challenge. Bu:Bw ratios greater than or equal to the Bu:Bw of the respective non-vaccinated/non-challenged group minus 2 standard deviations were associated with protection.

Statistical analysis. Bursal lesion scores pre-challenge were analyzed by unpaired t-test (GraphPad Prism, v8). Bu:Bw ratios and bursa scores post-challenge were analyzed by One-Way-ANOVA with uncorrected Fisher's LSD (Graphpad Prism, v8). Significant differences among groups were determined at p values lower than 0.05.

RESULTS

Vaccine detection. By using the Viral Flex Seq technology, sequencing of an amplified DNA fragment unique to the recombinant HVT-ND-IBD virus corroborated the presence of the vaccine in feather pulps and spleens from vaccinated birds.

Bursal lesions. Pre-challenge, at 14 days of age, bursa tissues in the different treatment groups were considered normal. Due to the normal variation observed at this age, scoring of bursal atrophy was not performed. Pre-challenge, at 18 days of age, bursa tissues were also found normal with mean scores between 1.2 and 1.4 (Figures 1A and 1B). No significant differences in bursal lesion scores were observed among treatment groups challenged with the Delaware E or AL-2 IBD viruses at 18 days of age. Bursal lesion scores in Delaware E and AL-2 challenged birds, as well as the non-vaccinated/non-challenged control group are presented in Figures 2A and 2B.

Delaware E challenge. The most severe bursal atrophy (score 4) was observed in the non-vaccinated/challenged group, at both 14- and 18-days challenge. Significantly lower bursal atrophy was observed in the HVT-ND-IBD vaccinated group, with mean bursal lesion scores of 2.58 and 2.37 in the groups challenged at 14 and 18 days of age, respectively. As expected, the lowest mean bursal lesion scores were observed in the non-vaccinated/non-challenged group, with mean scores of 1.92 and 2.04 for the 14- and 18-days challenge, respectively.

AL-2 challenge. Like the results obtained with the Delaware E challenge, severe bursal atrophy was also observed in the non-vaccinated/challenged group, with the highest mean bursal lesion scores of 3.96 and 4 for the 14- and 18-days challenge, respectively. Significantly lower bursal atrophy was observed in the HVT-ND-IBD vaccinated group, with mean bursal lesion scores of 2.92 and 2.93 for the 14- and 18-days challenge, respectively. Since the non-vaccinated/non-challenged group remained as the control group for both, the Delaware E and AL-2 challenges, mean bursal lesion scores of 1.92 and 2.04 were observed for the 14- and 18-day old challenge, respectively.

Bursa to body weight ratios. Mean Bu:Bw ratios with their standard deviations for the Delaware E and AL-2 challenged groups, as well as the non-vaccinated/non-challenged group, are summarized in Figures 3A to 3D. Reference Bu:Bw values to determine the efficacy of the HVT-ND-IBD vaccine to

protect against the challenge viruses at 14 and 18 days of age were 0.24 and 0.22, respectively.

Delaware E Challenge. High mean Bu:Bw ratios were observed in the non-vaccinated/non-challenged and HVT-ND-IBD vaccinated/challenged groups. No significant differences in mean Bu:Bw ratios were observed between those two groups at any of the two challenge ages. In contrast, significantly lower Bu:Bw ratios were observed in the non-vaccinated/challenged groups. Bu:Bw ratios in the HVT-ND-IBD vaccinated group at 14 (0.48) and 18 (0.54) days challenge were above the respective reference values of 0.22 and 0.24, indicating protection of vaccinated birds against bursal atrophy.

AL-2 Challenge. Like the Delaware E challenge, high mean Bu:Bw ratios were observed in the non-vaccinated/challenged and HVT-ND-IBD vaccinated/challenged groups. However, significant differences in mean Bu:Bw ratios were observed between those groups at both challenge ages. Significantly lower Bu:Bw ratios were also observed in the non-vaccinated/challenged group. Bu:Bw ratios in the HVT-ND-IBD vaccinated group at 14 (0.36) and 18 (0.44) days challenge were above the respective reference values of 0.22 and 0.24, indicating protection of vaccinated birds against bursal atrophy.

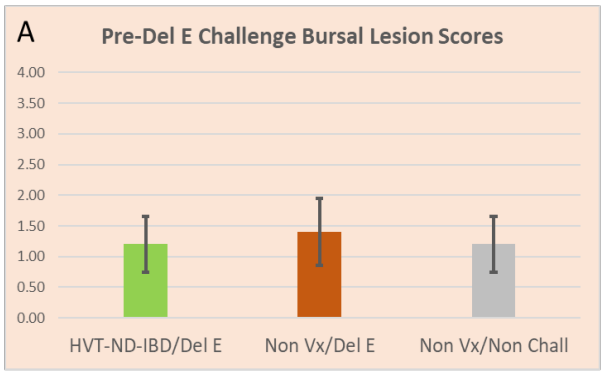
DISCUSSION

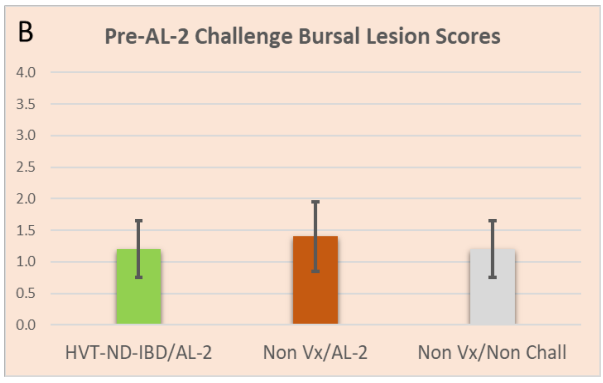
This study was designed to evaluate the level of protection provided by a recombinant HVT-ND-IBD vaccine (Innovax ND-IBD) administered subcutaneously to one-day old SPF chickens against ocular challenge at 14 or 18 days of age with the Delaware E and AL-2 variant IBD strains. In affected broiler flocks, these viruses have been associated with the presence of bursal atrophy and poor performance. When evaluated seven days post-challenge, severe bursal atrophy (mean bursal lesion scores between 3.96 and 4) was observed in the non-vaccinated group challenged at 14 and 18 days of age with both viruses. In contrast, the HVT-ND-IBD vaccinated/challenged and non-vaccinated/non-challenged exhibited significantly lower bursal lesion scores. Protection against challenged was determined using a reference bursa to body weight ratio, calculated by subtracting two standard deviations from the mean bursa to body weight ratio of the non-vaccinated/non-challenged group. Based on this criterion, the HVT-ND-IBD vaccine provided protection against early challenge with the Delaware E and AL-2 variant IBD viruses.

ACKNOWLEDGMENTS

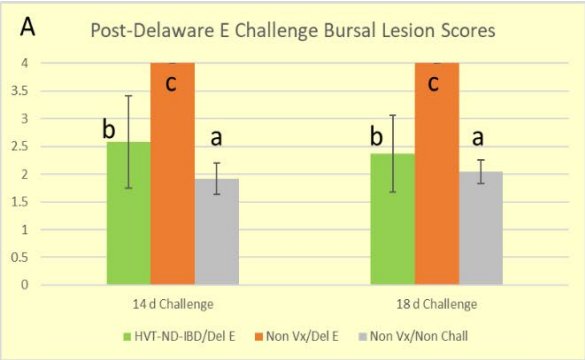
Special acknowledgment to Dr. Monique França for her contribution in the histopathological evaluation of bursa tissues and Eric Linnemann for his collaboration during the development of this study

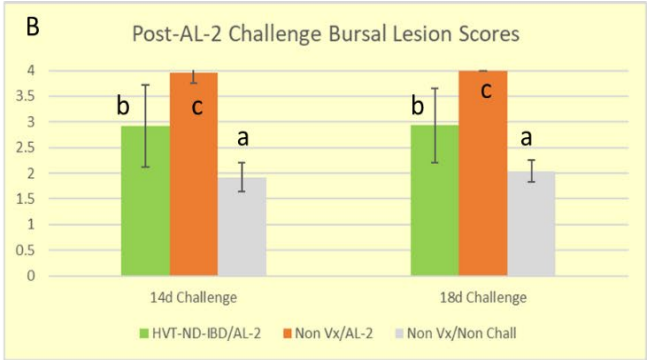
Figures 1A and 1B. Mean bursal lesion scores at 18 days of age (pre-challenge). The treatment groups were as follows: HVT-ND-IBD vaccinated/challenged (green bars), non-vaccinated/challenged (orange bars) and non-vaccinated/non-challenged (gray bars). The bursal lesion scoring system is based on scores of 1 to 4, as follows: 1 = normal to 10% follicular atrophy; 2 = 10% to 30% follicular atrophy; 3 = 30% to 70% follicular atrophy; 4 = more than 70% follicular atrophy. Vertical black lines represent the standard deviation for each treatment group.



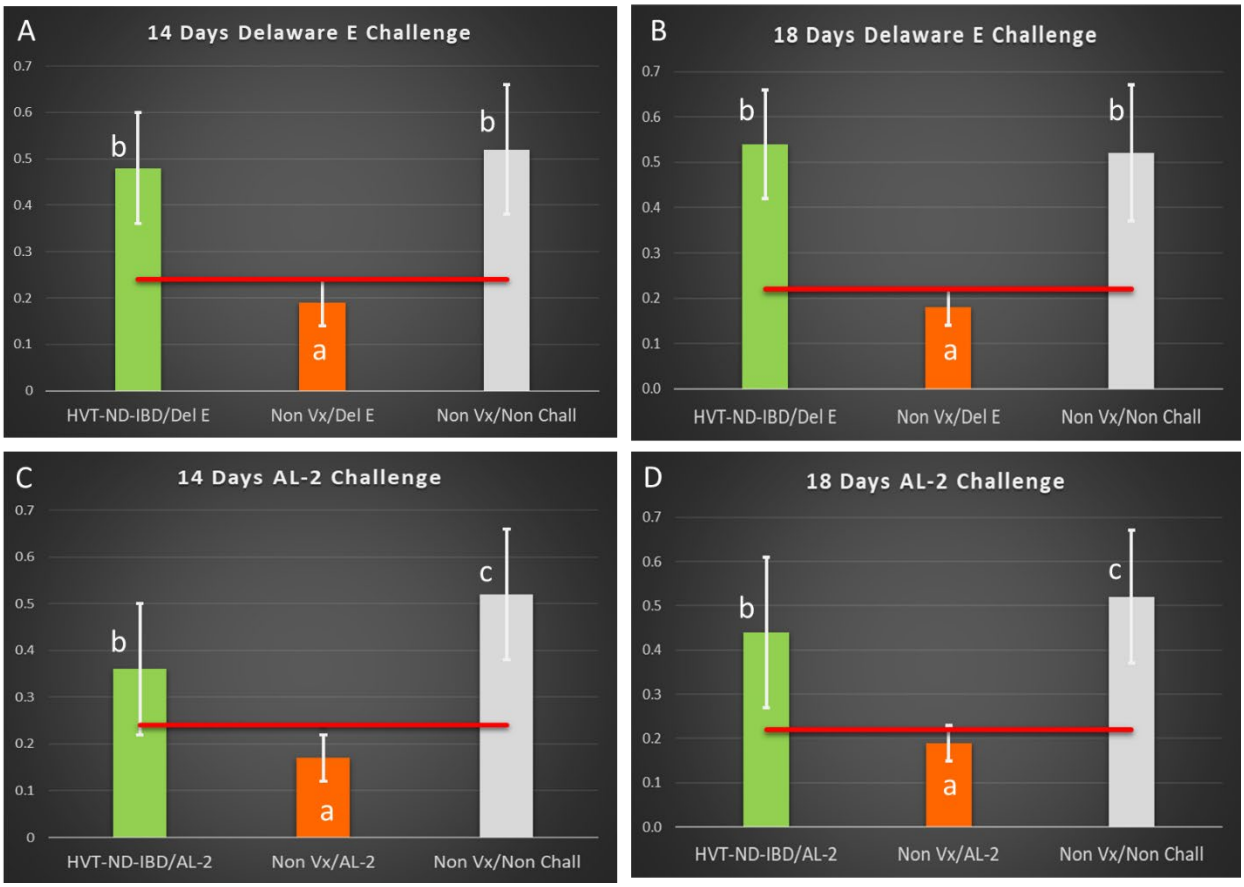


Figures 2A and 2B. Mean bursal lesion scores from birds challenged at 14 and 18 days of age with the Delaware E and AL-2 variant IBD viruses. Histopathological evaluation was performed seven days post-challenge (21 and 25 days of age, respectively). The treatment groups were as follows: HVT-ND-IBD vaccinated/challenged (green bars), non-vaccinated/challenged (orange bars) and non-vaccinated/non-challenged (gray bars). The bursal lesion scoring system is based on scores of 0 to 4, as follows: 1 = normal to 10% follicular atrophy; 2 = 10% to 30% follicular atrophy; 3 = 30% to 70% follicular atrophy; 4 = > 70% follicular atrophy. Vertical black lines represent the standard deviation for each treatment group. Different letters in the bars represent groups with significant differences at $p < 0.05$.





Figures 3A, 3B, 3C and 3D. Mean bursa to body weight ratios in birds challenged at 14 and 18 days of age with the Delaware E and AL2 IBD strains at seven days post challenge (21 and 25 days of age, respectively). The treatment groups were as follows: HVT-ND-IBD vaccinated/challenged (green bars), non-vaccinated/challenged (orange bars) and non-vaccinated/non-challenged (gray bars). The white vertical lines represent the standard deviations in each group while the horizontal red line represents the reference value for protection (values above the line associated with protection against challenge). Different letters in the bars represent groups with significant differences at $p < 0.05$.



STABILITY EVALUATIONS OF INFECTIOUS BRONCHITIS AND *SALMONELLA* VACCINES IN COMBINATION WITH DIFFERENT COCCIDIAL VACCINES

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SUMMARY

A series of studies were conducted to describe the impact of mixing with different coccidial vaccines on the stability of infectious bronchitis virus (IBV), Newcastle disease (NDV) vaccines, as well as in the stability and colonization of a *Salmonella* Typhimurium live vaccine. In experiment 1, one monovalent (Mass-type), one bivalent (Mass+Ark types) IBV, and one NDV vaccines were titrated by themselves or in combination with coccidial vaccine A (Coccivac B52) at different time points after reconstitution and mixing. There was no negative effect on the titer of the three vaccines with the addition of the coccidial vaccine, only a minimal reduction in the NDV vaccine was observed at two hours and at three hours in combination with the coccidial vaccine. In experiment 2, the stability of one live attenuated IBV Mass type and *S.* Typhimurium vaccines in combinations with three different coccidia vaccines: coccidial vaccine A, coccidial vaccine B, and coccidial vaccine C was evaluated by titration at different time points after reconstitution and combination. A minimal titer reduction of the IBV vaccine was observed in combination with cocci C after two hours at room temperature. However, the *Salmonella* vaccine showed significant titer drops after two hours (1 $\log_{10}$), and four hours (3 $\log_{10}$) also when it was combined with cocci vaccine C. Experiment 3 was an *in vivo* study conducted in one-day old chicks spray vaccinated with the same *Salmonella* vaccine in combination with two coccidial vaccines: coccidial vaccine A, and coccidial vaccine D. The Salmonella vaccine was re-isolated from chicks that received the combination with cocci A, but no re-isolation was obtained from chicks vaccinated in combination with coccidial vaccine D. The effect of the inclusion of potassium dichromate in vaccines C and D is discussed.

INTRODUCTION

Infectious bronchitis is an important respiratory disease of poultry, induced by a *Gammacoronavirus.* This virus is ubiquitous in most parts of the world where commercial poultry is present and can spread rapidly in susceptible birds. In young growing chickens, IBV causes respiratory disease that may predispose to bacterial complications, and poor economic performance. In addition to the common affinity for the respiratory tract, IBV strains may target other system or organs such as the female reproductive system, the alimentary tract, or the kidneys.

The enzootic avian paramyxovirus 1 (APMV-1) circulation in commercial poultry in the United States are lentogenic. Infection with these strains cause a typically mild clinical disease form with primary involvement of the respiratory tract. Vaccines including lentogenic viral strains are commonly used to prevent respiratory disease in the field.

Among the control practices against *Salmonella* infections is the application of attenuated or modified-live *Salmonella* vaccines that include serotypes such as Typhimurium or Enteritidis, that induce immunity versus homologous challenge and few other serotypes.

The application of live attenuated vaccines to control respiratory viruses such as infectious bronchitis and Newcastle, is routinely administered at the hatchery at one day of age, using different spray cabinets. In the same way, the application of anticoccidial vaccines is done by coarse spray at the hatchery. These vaccination practices are widely done in commercial poultry because guarantees the mass vaccine application before the birds are exposed to field challenge. The simultaneous application of multiple vaccines including Salmonella, coccidiosis, and respiratory viruses in the same mix by coarse spray has become a common practice at the hatcheries because is operationally more efficient and convenient. However, there is very few information about the impact of these vaccine combinations on the viability determined by vaccine titers of *Salmonella* or respiratory virus vaccines.

This series of studies were conducted to describe the impact of mixing different coccidial vaccines on the stability of infectious bronchitis and Newcastle vaccines, as well as in the stability and colonization of a commercially available *Salmonella* Typhimurium live attenuated vaccine.

MATERIALS AND METHODS

Experiment 1: **Stability infectious bronchitis and Newcastle live attenuated vaccines in combination with one coccidial vaccine.** The goal of this experiment was to evaluate the viability of two infectious bronchitis and NDV live attenuated vaccines in mixture with a live oocyst vaccine, (Coccivac B52®).

Vaccines:

1. Monovalent infectious bronchitis vaccine. Live attenuated infectious bronchitis vaccine, with a Mass type (Ma5) strain. (Mildvac Ma5, Merck Animal Health)
2. Bivalent infectious bronchitis vaccine with Mass and Ark types, live virus. (Mildvac Mass+Ark, Merck Animal Health)
3. Newcastle disease vaccine, with B1 type (C2) strain (Newhatch-C2, Merck Animal Health)
4. Coccidial vaccine A Live oocysts vaccine, with anticoccidial-sensitive strains of *Eimeria acervulina*, *E. maxima*, *E. maxima* MFP, *E. mivati* and *E. tenella* (Coccivac B52®, Merck Animal Health)

The infectious bronchitis, and Newcastle vaccines were diluted and mixed with the coccidial vaccine according to dilution and mixing procedures for vaccination with a spray cabinet system (21ml/box). Vaccine combinations were maintained at room temperature (approximately 25 C). Vaccine mix aliquots were obtained at zero, one, two and three hours after reconstitution for titration using specific pathogen free (SPF) chicken embryonated eggs (Charles and River Inc.). Vaccine titers per dose were calculated by Reed and Muench method.

Experiment 2: Stability infectious bronchitis and Salmonella Typhimurium live attenuated vaccines in combination with three commercial coccidial vaccines. The goal of this study was to evaluate the stability of live attenuated vaccines against infectious bronchitis virus and *Salmonella* Typhimurium in a mix with three commercial coccidia vaccines by titrations at zero, one, two and four hours after reconstitution and mixing.

Vaccines:

1. IBV-Vac: Live attenuated vaccine with Ma5 strain.
2. ST-Vaccine: Live attenuated vaccine that contains a strain of *Salmonella* Typhimurium belonging to O-antigen serogroup B, with a titer of 2-4 x 10^7 CFU/dose.
3. Coccidial Vaccine A: Live oocysts vaccine, with anticoccidial-sensitive strains of *Eimeria acervulina*, *E. maxima*, *E. maxima* MFP, *E. mivati* and *E. tenella*. (Coccivac B52 Merck).
4. Coccidial Vaccine B: Live oocysts vaccine, with anticoccidial-sensitive strains of *E. acervulina*, *E. maxima* and *E. tenella*.
5. Coccidial Vaccine C: Coccidiosis vaccine for chickens containing sporulated oocysts of *Eimeria maxima*, *E. tenella* and *E. acervulina*. This vaccine includes potassium dichromate as preservative.

Infectious bronchitis, and Salmonella vaccines were diluted and mixed with the coccidial vaccines according to typical dilution and mixing procedures for vaccination with a spray cabinet system (21ml/box). Five different mixes were prepared as follows:

1. IBV-Vac
2. ST-Vaccine
3. IBV-Vac + ST-Vaccine + Coccidial vaccine A
4. IBV-Vac + ST-Vaccine + Coccidial vaccine B
5. IBV-Vac + ST-Vaccine + Coccidial vaccine C

Vaccine combinations were maintained at room temperature (approximately 25C). Aliquots were obtained from each vaccine mix at zero, one, two and four hours after reconstitution and mixing. Viral titrations were carried out in SPF chicken embryonated eggs and vaccine titers were calculated by Reed and Muench method. *Salmonella* colony forming units per vaccine dose were calculated for each mixture at the same timepoints.

Experiment 3: Evaluation of colonization of chicks by a live Salmonella Typhimurium vaccine alone and in combination with a two different live attenuated coccidial vaccines. The goal of this experiment was to evaluate the colonization in chickens of a *S.* Typhimurium live vaccine applied by coarse spray mixed with two different live *Eimeria* oocyst vaccines.

Vaccines.

1. ST-Vaccine: Attenuated vaccine with a strain of *Salmonella*. Typhimurium belonging to O-antigen serogroup B.
2. Coccidial vaccine A: Live oocysts vaccine, with anticoccidial-sensitive strains of *Eimeria acervulina*, *E. maxima*, *E. maxima* MFP, *E. mivati* and *E. tenella* (Coccivac B52®).
3. Coccidial vaccine D: Live oocysts vaccine with precocious strains of *Eimeria maxima*, *E. acervulina*, and *E. tenella*. This vaccine included potassium dichromate as preservative.

Vaccine stocks were reconstituted and administered following the recommendations of their manufacturers. Dye Red (Merck Animal Health) was used to verify the uniform distribution of the vaccine on the chickens after spray vaccination. Vaccine combinations were maintained at room temperature (approximately 25 C).

Birds. One hundred and twenty-one-day old commercial chickens obtained from a commercial hatchery plant were used in this study. The chickens were free of *Salmonella,* determined by the negative isolation from meconium samples obtained through chick box papers.

Chickens were vaccinated by coarse spray at day of age using the cabinet Spraycox II®, birds remained under bright light for 10 minutes to stimulate preening. Seven vaccination treatments were carried out as follows:

1. ST-Vaccine
2. ST-Vaccine followed by coccidial vaccine A
3. Simultaneous administration of ST-Vaccine and coccidial vaccine A after 1 hr. post resuspension.
4. Simultaneous administration of ST-Vaccine and coccidial vaccine A after 3 hours post resuspension.
5. Administration of ST-Vaccine followed by coccidial vaccine D.
6. Simultaneous administration of ST-Vaccine and coccidial vaccine D after 1 hr. post resuspension.
7. Simultaneous administration of ST-Vaccine and coccidial vaccine D after 3 hrs. post-resuspension.

Sample collection. Five days post-vaccinations, 15 chicks from each group were randomly selected, euthanized by cervical dislocation and target organs were collected under sterility conditions. Organ samples consisting of the 1) pooled whole spleen and liver, 2) pooled intestinal tract with contents, and 3) ceca with contents were processed for culture of vaccine and/or salmonellae organisms. Culture from tissues and identification of *Salmonella* Typhimurium on plate media were conducted as per manufacturer's recommendations. Number of chickens with positive isolation as expressed over total of chickens.

RESULTS

Experiment 1. Vaccine titer determinations of IBV vaccine with Ma5 strain alone and combined with coccidial vaccine A at different times after mix preparation are included on Table 1. The highest decrease in titer was 0.5 Log_{10} after two hours from reconstitution. Interestingly the mixture between Ma5 and coccidial vaccine A exhibited a higher titer in comparison with the IBV vaccine alone (plus 0.5 Log_{10}) in the titration after three hours of incubation. Vaccine titer determinations of bivalent IBV vaccine Mass+Ark alone and combined with coccidial vaccine A at different times after mix preparation are included on Table 2. A decrease in titer of 0.5 Log_{10} at zero hours after reconstitution was observed.

Vaccine titer determinations of NDV vaccine (C2 strain) alone and combined with coccidial vaccine A at different times after mix preparation is included on Table 3. The mixture with NDV vaccine and coccidial vaccine A exhibited higher titers in comparison with the NDV vaccine alone after zero hours (difference of 0.66 Log_{10}) and one hour (difference of 0.80 Log_{10}) after reconstitution. A minimal reduction with the mixture was observed at 2 hours (-0.13 Log_{10}) and at 3 hours (-0.19 Log_{10}) in comparison with the NDV vaccine titrated alone.

Experiment 2. Vaccine titers expressed in $EID_{50\%}$/dose for the IBV vaccine alone and in combination with three commercial coccidial vaccines at different time points are shown in Table 4. According to these results, there was no remarkable reduction on the titers of the IBV vaccine when mixed with cocci vaccine A and B. A minor titer reduction in the titers of the IBV vaccine was observed after two hours in a mix with cocci vaccine C.

Vaccine titers expressed in colony forming units (CFU) /dose of the ST-Vaccine diluted alone or in combination with the IBV vaccine and three coccidial vaccines at different time points are shown in Table 5. A significant reduction in the titers of the ST-Vaccine was observed and two and four hours in the mix with cocci vaccine C. When compared with the ST-vaccine alone. The reduction after two hours of reconstitution was around one logarithm (from 2.43 x 10^9 to 1.66 x 10^8 CFU/dose), and around three logarithms (from 2.91 x 109 to 3.6 x 106 CFU/dose) after four hours.

Experiment 3. In the birds that received the ST-Vaccine alone by spray, the vaccine was isolated from cecal contents from only one bird (6.67%) and from intestinal contents of three birds (20%). No isolation was obtained from pool of liver and spleen. Regarding birds that received the ST-Vaccine alone and immediately the coccidial vaccine A in a separate solution, colonization of ST-Vaccine was observed in liver/spleen pool of one bird (6.67%), in the cecal contents of thirteen birds, (86.67%) (P<0.0001), and from intestinal contents of five birds (33.33%). No isolation was obtained from any bird that received cocci vaccine D in a separate dilution.

Birds that received simultaneously ST-Vaccine and cocci vaccine A after one hour from reconstitution, colonization of the Salmonella vaccine was observed in two birds (13.33%) in both the

liver/spleen pool and cecal contents, and from the intestinal contents of seven birds. No colonization was observed in the birds that received the same mix after three hours post-reconstitution.

No colonization of ST-vaccine was observed in the birds that received any treatment that included the cocci vaccine D at either one or three hours after reconstitution.

DISCUSSION

The titers of the IBV vaccine titrated alone are typical and within the specifications included in the Code of Federal Regulations, 9 CFR 113.327 for infectious bronchitis vaccines, and in 9 CFR 113.329 regarding Newcastle vaccine. The number of CFU per dose observed with the ST-Vaccine observed in this project were higher in comparison with the range specified for this vaccine. The manufacturer of the ST-Vaccine used in these studies, indicates a minimal titer of 2-4 $x10^7$ CFU per dose. This difference may have been due differences in the titration protocols.

According to the results of Experiment 1, the addition of the live oocysts coccidial vaccine A did not induce remarkable adverse effects on the titer and viability of the live attenuated monovalent IBV (Ma5), the bivalent IBV (Mass+Ark), and the NDV (C2) vaccines, when these vaccines were reconstituted and mixed with this coccidial vaccine and titrated after zero, one, two or three hrs. at room temperature.

Experiment 2 showed no significant reduction of the IBV vaccine (Ma5 strain) titers with the mixtures with coccidial vaccine A, and coccidial vaccine B. A mild reduction in the titers was observed with coccidial vaccine C after 2 hours at room temperature. This may suggest that the curve of titer reduction of the Ma5 vaccine through time might be faster when combined with coccidial vaccine C. However, the most remarkable effect of coccidial vaccine C was the significant reduction in the Salmonella vaccine observed after two and four hours.

Regarding Experiment 3, colonization of the vaccine *Salmonella* was determined by isolation of the vaccine from the immunized birds five days after the vaccination. Very few birds that received the vaccine alone showed colonization (only one bird through the isolation from the cecal contents and three birds through isolation from intestinal contents. This low isolation rate may be explained because only fifteen birds were sampled after five days. For future experiments it advised to increase the number of birds to be sampled and to increase the timepoints after vaccination. It is very possible that additional sampling at one and three days after vaccination may provide more information than only a sampling at five days post vaccination.

The application of coccidial vaccine A immediately after the application of the ST-Vaccine provided the best colonization rate; with 13 birds with positive isolation from cecal tonsils and five birds from intestinal contents. This procedure may have allowed better colonization because there was no direct impact by to the coccidial vaccine on the viability of the *Salmonella* vaccine. The simultaneous application of the ST-Vaccine with cocci vaccine A after one hour provided lower positive colonization in spleen/liver pool (2/15), cecal tonsils (2/15) and from intestinal contents (7/15).

No *Salmonella* colonization was observed in the birds that received the treatment with coccidial vaccine D, nor with all the birds sampled at three hours after reconstitution and mixing regardless of the treatment.

There is controversy on the successful combination of live *Salmonella* vaccines with coccidial vaccines due to the inactivation of this bacterium by the antagonistic additives in coccidiosis vaccines. Potassium dichromate ($K_2Cr_2O_7$) in solution is usually included to prevent fungi development, it prevents the growth of ciliated and non-ciliated protozoa (micro-flora) and some bacteria, and it is thought to promote sporulation of oocysts. Therefore, potassium dichromate is used as preservative agent in some *Eimeria* vaccines. The oocyst wall of *Eimeria* seems to provide sufficient protection to the parasite but $K_2Cr_2O_7$ is toxic to several microorganisms including *Salmonella*. In these series of studies, a drastic reduction of *S.* Typhimurium CFUs and a mild titer drop were observed when these vaccines were combined with coccidial vaccine C, that includes $K_2Cr_2O_7$. Moreover, no colonization of *S.* Typhymurium vaccine was observed in the chickens that received vaccine D that also includes $K_2Cr_2O_7$ in its formulation.

Therefore, when vaccine combinations that include coccidial, *Salmonella* and viral vaccines are prepared to be applied by coarse vaccinations in hatcheries, it is highly recommended to avoid including coccidial vaccines with potassium dichromate in these vaccine combinations. In the case of *Salmonella* and coccidial vaccines, it seems that the best practice is to apply them using separate nozzle systems, unless both coccidial and *Salmonella* vaccines can be administered within one hour after resuspension/mixing.

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Table 1. Vaccine titers in EID_{50} per vaccine dose of IBV monovalent vaccine (Ma5 strain) alone and combined with coccidial Vaccine A at different times after mix preparation.

Time of titration after reconstitution and mixing (Hrs.)	Monovalent IBV Vaccine (Ma5 strain) Log_{10}	Mix Monovalent IBV Vaccine/Cocci Vaccine A Log_{10}	Difference
Zero	3.94	3.82	-0.12
One	4.95	4.69	-0.26
Two	4.82	4.32	-0.5
Three	4.32	4.67	+0.35

Table 2. Vaccine titers in EID_{50} per vaccine dose of IBV bivalent vaccine (Mass+Ark serotypes) alone and combined with coccidial vaccine A at different times after mix preparation.

Time of titration after reconstitution and mixing (Hrs.)	Bivalent IBV Vaccine (Mass+Ark serotypes) Log_{10}	Mix Bivalent IBV Vaccine/Cocci Vaccine A Log_{10}	Difference
Zero	4.08	3.57	-0.51
One	3.32	3.84	+0.52
Two	3.82	3.82	None
Three	3.79	3.94	+0.15

Table 3. Vaccine titers in EID_{50} per vaccine dose of Newcastle vaccine (C2 strain) alone and combined with coccidial vaccine A at different times after mix preparation.

Time of titration after reconstitution and mixing (Hrs.)	NDV Vaccine (C2 Strain) Log_{10}	NDV Vaccine/Cocci Vaccine A Log_{10}	Difference
Zero	6.12	6.78	+0.66
One	6.68	7.48	+0.80
Two	7.03	6.90	-0.13
Three	6.32	6.13	-0.19

Table 4. Vaccine titers in EID_{50} per vaccine dose of IBV vaccine (Ma5 strain) alone and combined with three commercial coccidial vaccines at different times after mix preparation.

Time of titration after reconstitution and mixing (Hrs.)	IBV Vaccine (Ma5 Strain) Log_{10}	IBV Vac/Cocci Vaccine A Log_{10}	IBV Vac/Cocci Vaccine B Log_{10}	IBV Vac/Cocci Vaccine C Log_{10}
Zero	5.12	5.0	5.78	5.0
One	4.68	5.0	5.62	5.63
Two	5.31	5.46	5.57	4.87
Four	4.62	5.0	4.62	4.6

Table 5. Vaccine titers in colony forming units (CFU) per vaccine dose of ST-Vaccine alone and combined with IBV vaccine (Ma5) and three commercial coccidial vaccines at different times after mix preparation.

Time of titration after reconstitution and mixing (Hrs.)	ST-Vaccine	ST-Vaccine/IBV vac	ST-Vaccine/IBV vac/Cocci Vaccine A	ST-Vaccine/IBV vac/Cocci Vaccine B	ST-Vaccine/IBV vac/Cocci Vaccine C
Zero	3.04×10^{10}	2.59×10^{10}	2.68×10^{10}	3.16×10^{12}	1.63×10^{9}
One	1.94×10^{9}	2.26×10^{9}	2.13×10^{9}	2.01×10^{10}	1.72×10^{9}
Two	2.43×10^{9}	2.34×10^{11}	1.03×10^{9}	1.57×10^{9}	1.66×10^{8}
Four	2.91×10^{9}	1.38×10^{10}	1.59×10^{9}	3.22×10^{10}	3.6×10^{6}

DEVELOPMENT AND DETERIORATION OF COCCIDIA OOCYSTS IN WET AND DRY LITTER AT DIFFERENT TEMPERATURES

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SUMMARY

Litter management can help to reduce infection pressure with coccidia oocysts. We used a model in a laboratory setting to evaluate the environmental influence on sporulation and survival of coccidia oocysts. Two pilot experiments were conducted testing the influence of temperature and humidity on oocyst recovery, oocyst sporulation, oocyst size and oocyst deterioration. In the first experiment, comparing dry and wet litter kept at cool and warm temperatures, oocyst counts and the percentage of sporulated oocysts were higher in litter kept at cool temperatures. Differences between dry and wet litter were comparatively minor. In the second experiment, the influence of dry and wet litter kept under cool conditions was tested. There was no influence on oocyst size, but the proportion of damaged oocysts was slightly higher in wet litter. In the future, the model can be used to investigate the effect of litter treatments on the infection pressure with coccidia oocysts.

INTRODUCTION

Coccidiosis is one of the biggest challenges in poultry production due to its great potential to reduce the birds' performance and the resilience of the parasite's free form, the oocysts in the environment (1). The oocysts remain infectious in the environment for a long time, which contributes to their nearly universal occurrence in chicken flocks. Concomitantly, due to economic reasons, poultry litter is reused for several flocks in many countries (2). These two factors together require proper management of litter to reduce the infection pressure of coccidia.

In order to be infective, oocysts need to sporulate in the environment, and environmental conditions such as temperature, humidity and composition of the matrix can affect the sporulation of oocysts (3). Temperature seems to be the factor with the most obvious influence on oocyst development. In fecal suspension, the temperature allowing the oocysts to sporulate in the shortest time was about 30°C (4, 5). While this information is important for laboratory procedures, there is little relevance for poultry flocks, where oocysts are in litter, and temperature is set according to the needs of the chicks. However, heating houses and litter, e.g., by windrowing, between flocks might destroy oocysts, but no information on this aspect is available.

Litter moisture is often regarded as important parameter. It is also a parameter that is relatively easily controlled by adjusting ventilation rates. However, sporulation rates were not significantly different between litter with a relative humidity of 40 versus 80% (3). Only very dry litter with a relative humidity of 16% allowed for a higher sporulation rate compared to litter with a relative humidity of 62% (6). Sporulation rates are higher in litter than in pure feces, presumably due to higher concentrations of ammonia in feces (3). The addition of various litter treatments like superphosphate, meta-bisulfide, and charcoal reduced oocyst counts (7), while covering litter with plastic had no effect (2).

The aim of this project was to test a laboratory model that can be used in future projects to test the influence of different litter treatments on the infection pressure by *Eimeria* oocysts. For that, the environmental influence on sporulation and deterioration of coccidia was investigated in two pilot experiments. Investigated parameters were oocyst recovery, sporulation, size, and deterioration.

MATERIALS AND METHODS

The first trial compared cool and warm litter in wet and dry conditions. Two hundred grams of dry litter and 200 g of feces containing *Eimeria maxima* oocysts were mixed and divided into four aliquots, each stored in a plastic box, covered loosely with a lid. To two aliquots, 20 mL of tap water were added. One dry and one wet aliquot were stored 20 cm under a heat lamp adjusting the temperature to about 34°C, the other two aliquots were stored at room temperature, about 20°C. In four-day intervals, until the end of the experiment at 28 days, samples were collected and processed with routine procedures for counting in a McMaster chamber. When counting the oocysts, the percentage of sporulated oocysts was determined.

The second experiment tested the influence of moisture under cool conditions. Four hundred grams of dry litter and 400 g of feces containing *E. acervulina* oocysts were mixed and divided into eight aliquots,

each stored in a plastic box, covered loosely with a lid at room temperature. To four aliquots, 25 mL of tap water were added. All boxes were weighed. Every three days until the end of the experiment at 22 days, boxes were weighed, and water was added to the original weight to compensate for evaporation of water. On the same days, samples were collected and processed with routine procedures for counting in a McMaster chamber. In addition, photos of 50 different areas of the chamber were taken. In these photos, length and the width of 100 oocysts on days 1 – 10 and of 20 oocysts on days 13 – 22 were measured, and the size of the oocysts was calculated. Measured oocysts were classified as intact or damaged (Figure1).

Oocyst counts and sizes as well as the percentage of damaged oocysts were tested for significant differences between treatments ($P < 0.05$) by t-test using R 3.6.0 (8). Oocyst sizes were tested for differences between damaged and un-damaged oocysts by t-test using R.

RESULTS AND DISCUSSION

In the first experiment, the dry and wet aliquots kept at cooler temperature had the higher oocyst counts than the dry and wet aliquots kept under the heat lamp. However, the effect of the added water differed. At the cooler temperature, oocyst counts tended to be higher in the dryer aliquot, while at the warmer temperature the reverse effect was observed (Figure 1). The percentage of sporulated oocyst was constantly higher in the aliquots kept at the cooler temperature and reached 50% at the end of the experiment. In contrast, in the aliquots stored under the heat lamp, there was no systematic difference between the aliquots with and without added water. Only on one day more than 10% of the counted oocysts were sporulated (Figure 2).

The first experiment indicated that coccidia oocysts disintegrate slower in a cooler environment, while at the same time sporulate at a higher rate. For this reason, the second experiment was done only at the cooler temperature.

In the second experiment, the average oocyst counts were higher in the wet litter during the first ten days. Thirteen days after the start of the experiment, oocyst counts in the dry group were higher, later differences were not significant (Figure 3). In contrast, the percentage of damaged oocysts did not differ during the first ten days, while later the percentage of damaged oocysts was higher in the dry group (Figure 4A). Oocysts were, on most days, larger in the wet group. Damaged oocysts were in average smaller than intact oocysts (Figure 4B).

The results showed oocysts counts were initially higher in wet litter. However, there was also a high variability with seemingly increasing oocyst counts over time. It was surprising that the proportion of damaged oocysts was higher in dry litter. These suggest that, in dry litter, damaged oocysts might have taken a longer time to disintegrate completely after initial damage. Damage was also indicated by a smaller size compared to oocysts that looked intact.

Both trials indicate that coccidia oocysts behave different in different conditions. Also, oocyst counts, sporulation status and damage were shown to be suitable parameters. This information is essential in order to setup future studies that will test how different litter treatments influence the infection pressure by *Coccidia* oocysts.

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Figures 1 and 2. Coccidia oocyst counts per gram (OPG) of litter in litter spiked with *E. maxima* (Figure 1) and percentage of sporulated oocysts in litter spiked with *E. maxima* (Figure 2)

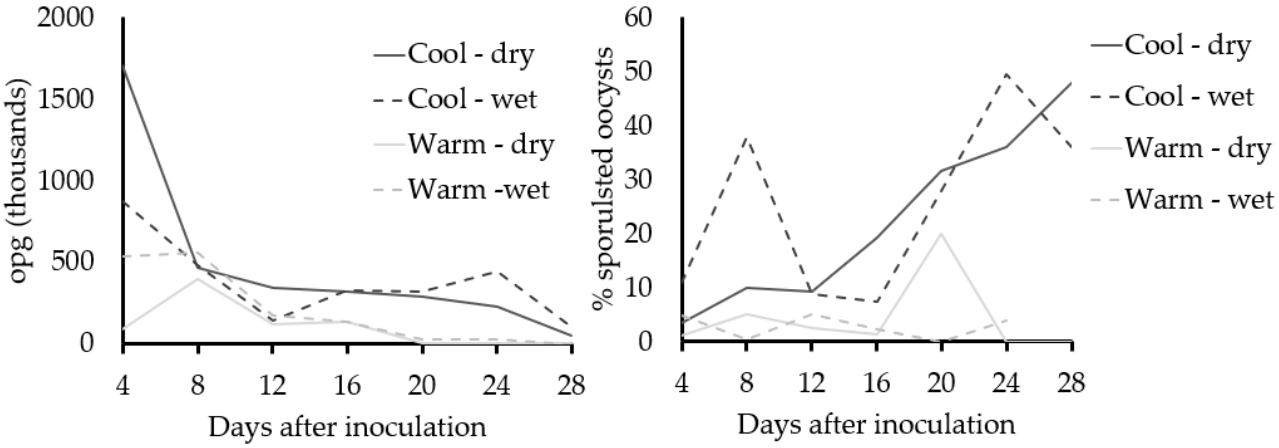


Figure 3. Coccidia oocyst counts per gram (OPG) of litter spiked with *E. acervulina*; error bars indicate standard deviation; stars indicate a significant difference.

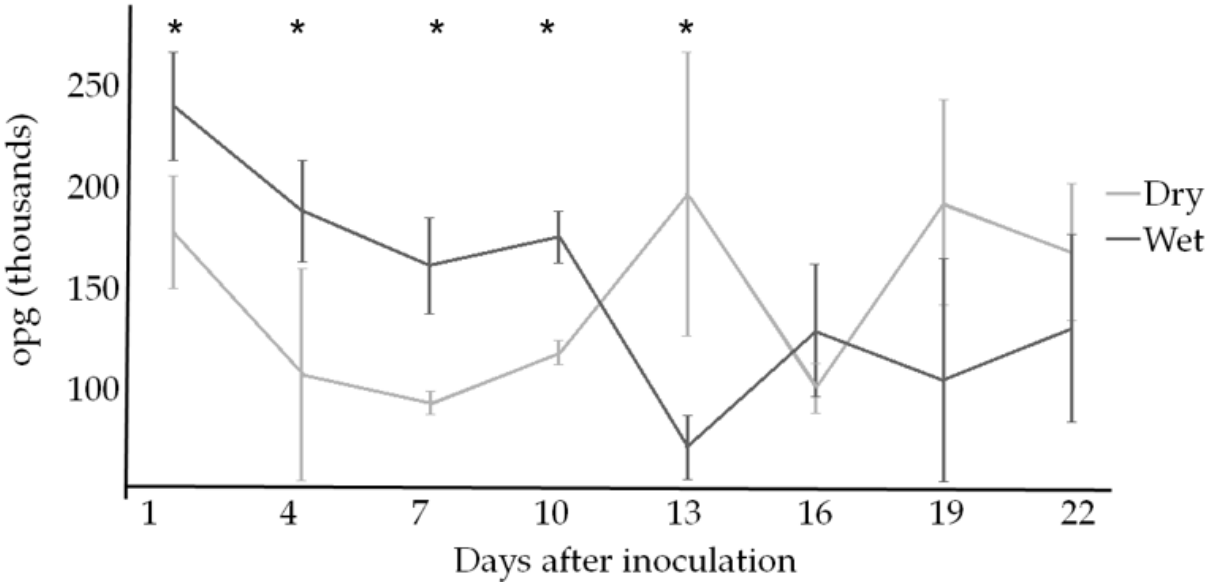
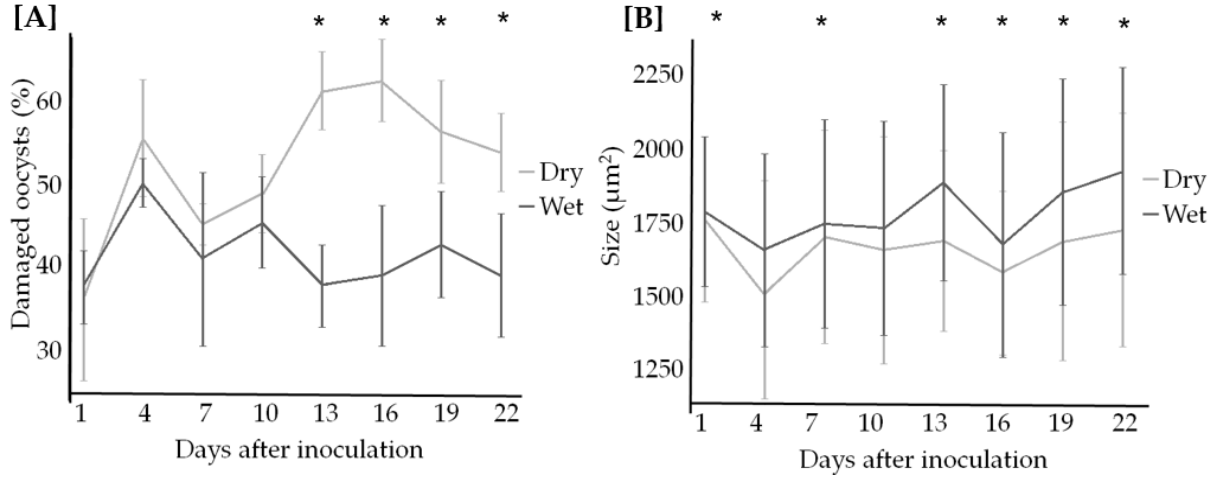


Figure 4. Proportion of damaged *E. acervulina* oocysts (A) and oocyst sizes (B) in dry and wet litter spiked with *E. acervulina;* error bars indicate standard deviation; stars indicate a significant difference.



INFECTIOUS BURSAL DISEASE VIRUS DYNAMIC, BURSA LESIONS AND IMPACT OF VACCINATION IN COMMERCIAL BROILER FARMS IN CANADA

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SUMMARY

Various types of infectious bursal disease (IBD) vaccines are employed and appear effective clinically. A field study was conducted to characterize the viruses that are circulating under various vaccination scenarios in commercial broiler flocks across Canada in 2020. We targeted farms with a history of IBD virus (IBDV) challenge, whether under control or not, and the vaccination programs in use were documented. Bursas were collected at two to three occasions in each flock and analyzed for histopathological lesion score and for the presence of IBDV by PCR and/or NGS. Bursa histopathology revealed acute bursitis at 25 days of age or later. The field viruses identified branch off the IBDV T1_strain_reference_genogroup 2. Vaccine viruses were not recovered from flocks that used field vaccinations. In conclusion, vaccination strategies have an important role in bursa health and immunocompetence.

INTRODUCTION

IBD is also called Gumboro disease and results in immunosuppression in young chickens worldwide. The disease is caused by IBDV, a double-stranded RNA birnavirus highly prevalent, stable in the environment, and resistant to many physical and chemical agents (1). The majority of the IBDV strains circulating in Canada are variant strains (2, 3, 4). Infected flocks suffer increased risk of secondary bacterial infections (4), higher rates of condemnations (5), and suboptimal performance. Common interventions used to manage IBDV include litter removal and cleaning between flocks, immunization of breeders to transfer maternal antibodies, and vaccination of the progeny. Success of the control program in the field is primarily assessed by monitoring end of flock serology trends.

MATERIALS AND METHODS

A study was conducted to characterize the circulating IBDV, whether bursal infection occurs, and when it occurs, under various vaccination scenarios in commercial broiler flocks across Canada (Nova Scotia, Quebec, British Colombia) in 2020. Four veterinarians selected farms from their clients with a history of IBDV challenge, whether under control or not, and the vaccination programs were documented. Each veterinarian was associated with a different hatchery and selected three flocks. Each had a different set of motivations to join the study and the sampling template varied slightly based on individual's specific perspective and interests. For tissue collection four healthy birds were randomly selected and euthanized at each sampling point. For serology 10-12 healthy birds were randomly selected and released at each sampling point. Bursa, thymus and serum were collected on at least two occasions in each flock. Bursa were analyzed for histopathological lesion score characterized as varying degrees of lymphoid depletion and follicular restitution and for the presence of IBDV by PCR and/or next generation sequencing (NGS) (Viral Flex-Seq® IBD virus test by Rapid Genomics, LLC). Thymus histopathological lesion were scored to assess immunocompetence. Serum were submitted for IBD serology using the IBD-XR ELISA (IDEXX). Feather pulp samples were collected from 20 birds per flock at approximately 25 days of age when rHVT-ND-IBD vaccine (Innovax®-ND-IBD, Merck Animal Health, Kirkland, QC, Canada) was used. These samples were used to validate the presence of the unique Innovax® HVT vector using NGS.

RESULTS

Bursa histopathology revealed acute bursitis at 25 days of age or later in vaccinated flocks (Table 1). Maternal immunity alone pushed acute bursitis lesions to 27 days in one flock of case 1. In this case the

competitor rHVT-IBD vaccine used only delayed acute bursitis by three days to 30 days of age.

Acute bursitis was not observed in three of the flocks from cases 1, 2, and 3. Two of these had received rHVT-ND-IBD (Innovax®-ND-IBD) *in ovo*; no virus was recovered, and end of flock titers were low.

IBDV was recovered and sequenced from all bursa with acute bursitis. The field viruses identified all branch off the IBDV T1_strain_reference_genogroup 2 labeled as T1-like for simplification in Table 1.

Live vaccine viruses were not recovered except for one flock in case 2 that had received a live virus-antibody complex vaccine *in ovo*. This flock had acute bursitis at 25 days caused by the Winterfield 2512 vaccine virus (Table 1 & 2). Bursa follicular restitution and severe thymus lesions were suggestive of lower immunocompetence in this flock.

All flocks from case 4 received the same live vaccine at 14 days and acute bursitis was observed the earliest in the flock that had higher maternal antibodies (Tables 1 and 2).

The presence of the unique Innovax® HVT vector was confirmed in every flock sampled in Cases 2 and 3 (Table 3). The presence of more than one HVT was observed in three flocks indicating that hatcheries that custom vaccinate for each customer are prone to residual vaccine cross-contamination.

DISCUSSION

This survey highlights the limits of relying only on serology to monitor IBD control in commercial broiler operations. A panel of tests that include vaccine administration validation, immunocompetence assessment using histopathology, virus identification and serology can provide a more complete and accurate view of the IBDV dynamic and control.

Despite the multiple variables inherent to such field survey such as different breeder sources and immunization, four different hatcheries, three provinces across four time zones, similar field T1-like IBDV parents to the Delaware E IBDV variant were found in all four cases. More refined analysis beyond the scope of this survey would possibly reveal differentiations between strains.

We learned that various vaccination strategies can indeed be effective or partially effective. However, results are influenced by adequate vaccine administration. Assessing and monitoring vaccine application will ensure that organizations get what is expected out of vaccines and true failures be differentiated from administration errors. Accidental concurrent administration of a second HVT or rHVT may impair replication of the desired HVT vector vaccine and results in lower protection. In Case 3 (Table 1 & 3) flock with lowest residual HVT cross-contamination had better IBDV control.

Protection from vaccinations with modified live vaccines is influenced by maternal immunity. Still, one flock vaccinated in hatchery with a traditional modified live virus (MLV) vaccine did fare as well in terms of timing of acute bursitis as flocks that received MLV in the field and better than the flock that received a virus-antibody complex vaccine in hatchery. Recovering the Winterfield 2512 indicates that the vaccine virus was successful in displacing the field virus but caused more severe bursa and thymus lesions than the field virus. It is interesting that the two different IBDV from the MLV field vaccines couldn't be recovered. It is possible that maternally derived antibodies (MDA) were too high at the time of vaccination and didn't get a good multiplier effect to find the vaccine virus in the field. Meat withdrawal of 21 days on all MLV vaccines limits their use after 12-14 days of age. This problem will accentuate in the future given that birds reach the desired market weight sooner with ongoing improvements in genetics and husbandry.

Vaccination strategies have an impact on bursa health and immunocompetence. Under the conditions of this field study bursa were within normal and field virus not found in three flocks. Two of these received rHVT-ND-IBD alone or with 89/03® (Merck Animal Health, Kirkland, QC, Canada).

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Table 1. Vaccination program, age when acute bursitis was observed, associated virus, and end-of-flock IBDV titer

Case #	Hatchery vaccine	Hatchery vacc route	Field vaccine	Field vacc route	field vacc age - days	acute bursitis age - days	IBD PCR CT	IBD virus	IBD XR Gmean	last sampling age - days
1	rHVT-IBD	*in ovo*	0			30	17.63	T1-like	175	30
	0		0			27	20.92	T1-like	1781	30
	0		MLV-1	CS	9	> 30	neg		9	30
2	rHVT-ND-IBD & 89/03®	*in ovo*	MLV-1	DW	10	> 35	neg		n/a	35
	0		MLV-1	DW	10	25	n/a	T1-like	7735	35
	virus-antibody complex	*in ovo*	MLV-1	DW	10	25	n/a	Winterfield 2512	n/a	35
3	MLV-3	CS	0			33	21.12	T1-like	6187	33
	rHVT-ND-IBD	*in ovo*	0			> 33	34.78		95	33
	rHVT-ND-IBD	*in ovo*	0			27	24.42	T1-like	5260	36
4	0		MLV-2	CS	14	26	n/a	T1-like	6339	35
	0		MLV-2	CS	14	31	n/a	T1-like	4543	34
	0		MLV-2	CS	14	34	n/a	T1-like	847	34

®Merck Animal Health, Kirkland, QC, Canada; CS = coarse spray; DW = drinking water

Table 2. Bursa & Thymus score for case 2 & 4 to illustrate range of observations.

				Bursa Score								Thymus Score							
Case #	Hatchery vaccine	Field Vaccine	Age (days)	1	2	3	4	5	6	Mean	Comment	1	2	3	4	5	6	7	Mean
2	rHVT-ND-IBD & 89/03®	MLV-1 @10D	15	0	0	0	0			0.00	Within normal limits	0	0	0	0	0	0	0	0.00
			25	0	0	0	0	0		0.00	Within normal limits	0	0	0	0	0	0	0	0.00
			35	0	0	0	0			0.00	Within normal limits	0	0	0	0	0	0	0	0.00
	0	MLV-1 @10D	15	0	0	2	0	0	0	0.33	Nonspecific depletion/stress	0	0	0	0	0	0		0.00
			25	4	5	0	4			3.25	Acute bursitis/IBD	0	0	0	0	2	0		0.33
			35	2	3	2	3			2.50	Recovery IBD	0	0	1	0	1	0		0.33
	virus-antibody complex	MLV-1 @10D	15	0	0	1	0	0	0	0.17	Nonspecific depletion/stress	0	0	0	0	0	0		0.00
			25	5	5	0	0			2.50	Acute bursitis/IBD	0	0	0	0	0	0		0.00
			35	4	4	4	4			4.00	Recovery IBD	0	4	3	4	3	3	0	2.43
4	0	MLV-2 @14D	15	0	0	0	0			0.00	Within normal limits	0	0	0	0				0.00
			26	4	5	5	0			3.50	Acute bursitis/IBD	0	0	0	0				0.00
			35	5	4	4	3			4.00	Recovery IBD	0	2	0	0				0.50
	0	MLV-2 @14D	15	0	0	0	0			0.00	Within normal limits	0	0	0	0				0.00
			26	0	0	0	0			0.00	Within normal limits	0	0	0	0				0.00
			35	4	4	5	4			4.25	Recovery IBD	0	3	2	2				1.75
	0	MLV-2 @14D	13	0	0	0	0			0.00	Within normal limits	0	0	0	0				0.00
			25	0	0	0	0			0.00	Within normal limits	0	0	0	0	0			0.00
			34	5	5	5	5			5.00	Acute bursitis/IBD	0	0	2	3				1.25

Table 3. Viral Flex-Seq® vaccine test results

Case #	Hatchery vaccine	Route	hatchery vaccine evaluation
2	Innovax®-ND-IBD & 89/03®	*in ovo*	4/20 with non Innovax HVT contamination
3	MLV-3	CS	4/28 with Innovax® contamination
3	Innovax®-ND-IBD	*in ovo*	1/28 with non Innovax HVT contamination
3	Innovax®-ND-IBD	*in ovo*	2/28 with non Innovax HVT contamination

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

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SUMMARY

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

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

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

INTRODUCTION

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

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

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

How is the sensitivity determined? Sensitivity can be determined in various ways, some have suggested by comparing differences in body weight gain between the treated group and the negative and positive controls (8), while others have suggested excretion of oocysts (3), an anticoccidial index (8) or body weight gain and feed conversion (10). However, since anticoccidial drugs are designed to prevent

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

SAMPLE COLLECTION AND SUBMISSION

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

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

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

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

BRIEF DESCRIPTION OF THE TEST

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

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

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

OTHER CONSIDERATIONS

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

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

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

CONCLUSION

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

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EGG DROP SYNDROME: DIAGNOSTIC CHARACTERIZATION OF A STRAIN IN US CHICKENS

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SUMMARY

Egg drop syndrome, caused by duck atadenovirus A in the *Adenoviridae* family, is characterized by a noticeable drop in egg production as well as production of soft-shelled eggs in otherwise healthy birds. While duck atadenovirus A has a worldwide distribution in geese and ducks, clinical cases are reported from Europe, Asia, Latin America, and Africa in ducks, chickens and other avian species. The virus has been detected recently in layer chickens in the United States exhibiting clinical disease. The ability to identify, grow, and characterize this virus is paramount in controlling its spread. Several methods of culturing and inactivation were evaluated, as well as other diagnostic tests such as HA, PCR and sequence analysis. Being able to grow, characterize, and inactivate EDS will assist in identifying infected flocks and provide an avenue for biologic intervention.

INTRODUCTION

Egg drop syndrome is caused by duck atadenovirus A (DAdV-1) in the *Adenoviridae* family in the *Atadenovirus* genus (1). Other names include egg drop syndrome-76 (EDS-76) and adenovirus 127, which was first identified in Northern Ireland in 1976. Waterfowl are the natural hosts, including domesticated ducks and geese and found worldwide. Characteristic symptoms include a noticeable drop in egg production as well as the production of soft-shelled eggs in otherwise healthy birds. The main route of transmission is vertically through the egg, though it can be spread by contaminated water and other fomites as virus is shed in the feces (2). These clinical cases have been reported in chickens, turkeys, and quail and antibodies have been found in numerous avian species (3). The majority of clinical cases are reported in Europe, Africa, Asia and Latin America and, until recently, there were no documented cases in the United States since the 1970s (2). The following report details the case history and laboratory analysis of the recent re-emergence of DAdV-1 in the United States layer chicken population.

CASE REPORT

Case history. In July of 2018, a producer in Pennsylvania noticed a large increase in the number of soft shell or shell-less eggs in their layer facility. Pale to white eggs were also common, but no gross pathology was found upon posting birds (Figures 1a and 1b). After a tentative diagnosis of a nutritional or copper exposure related problem, the birds did not recover and the problem was spreading to other floors in the lay house. The presence of DAdV-1 in two linked sites in PA was confirmed by hemagglutination inhibition testing at the National Veterinary Services Laboratory (NVSL). The affected eggs were found in various brown breed layers. To date, white birds with a known exposure have not shown these symptoms. Age of onset can vary but typically occurs after the birds are moved into the lay house and coming into production (20-30 weeks of age). Since the DAdV-1 diagnosis, all non-vaccinated brown flocks in this facility have shown similar breaks. DAdV-1 has also been identified at three other locations in PA with similar symptoms.

Virus isolation. An initial isolation attempt was unsuccessful due to the presence of fowl adenovirus type 1. New samples were submitted and DAdV-1 virus was isolated in November of 2019 in duck embryo fibroblasts (DEF) cells from the shell gland of a brown layer. Disease was confirmed and reproduced in brown hens by either feeding 24-week old hens affected eggs and by inoculating 27-week old hens with DAdV-1 virus propagated in DEFs. Pale eggs were observed in both cases. Multiple other culture methods were tried on isolated virus, including passage in the following immortalized cells: swine testicle cells, porcine kidney cells, Vero cells, and leghorn male hepatoma cells. No cytopathic effect was noted and hemagglutination assay (HA) and PCR confirmed no growth. Chicken embryonic fibroblast (CEF) and embryonated chicken eggs also failed to propagate the virus. Chicken embryonic liver cells were successful at growing the DAdV-1 virus, though CPE was subtle or not easily detected. The most efficient replication occurred in embryonated duck eggs. No lesions were evident in the embryos but the HA on chorioallantoic sac (CAS) fluid showed the virus grew to a high HA titer of >100,000 HA units.

Chicken blood yielded higher HA values than turkey red blood cells.

Molecular characterization. Samples were extracted using the MagMax Core Nucleic Acid Purification Kit (Life Technologies). A quantitative real-time PCR (qPCR) from a previously published paper was used to confirm the growth of DAdV-1 (4). One of the positive samples was then sequenced on the Illumina MiniSeq and raw reads were normalized and trimmed using BBnorm (5), and Trimmomatic (6). Curated reads were aligned to NCBI reference strain 127 (GenBank NC001813) with MiniMap 2 (7).

Phylogenetic analysis. A multiple sequence alignment was performed with clustal Omega and trees were built in Bionumerics using maximum parsimony with 1000 replicates (Fig2a-d) (8). The complete genome is >99.7% similar to 7 of the 8 DAdV-1 strains analyzed and showed least homology at 98.1% to strain FMV-19-2234581 from ducklings in Canada (GenBank MN310513). The fiber protein showed the highest diversity with the Canada strain only sharing 92.3% identity with the recent U.S. strain.

Inactivation. In order to produce a traditional killed vaccine, inactivation of the antigen must be completed. Two different methods were used to inactivate DAdV-1 successfully. One method was the use of binary ethylenimine and the other was the use of 10% formalin at 0.4% v/v. Future studies should be done to determine which inactivation method best preserves the antigenic structure of the virion to illicit the most protective immune response. To ensure inactivation of the antigen was complete, the inactivated antigen was passaged two times in embryonated duck eggs and HA activity continually decreased. qPCR values from each passage also indicated no viral growth during passage.

DISCUSSION

There are currently no commercial vaccines in the United States for egg drop syndrome caused by DAdV-1. Some challenges to producing an autogenous vaccine in the U.S. include the lack of specific pathogen free (spf) duck embryos as well as the costs associated with that method of vaccine production. Production of antigen in CEL or DEF cells is also cost prohibitive. Another issue is the risk of contamination from using non-spf sources and the costs of additional testing. A surveillance program is being implemented to track any potential future spread of DAdV-1 throughout U.S. flocks.

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Figure1.

1(a) Pale, soft eggs amid normal brown eggs

1(b) soft, shell-less eggs

Figure 2.

2(a) Whole Genome Maximum Parsimony

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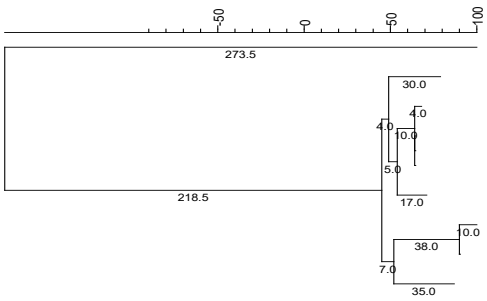

FMV-19-2234581/duck/Canada/2019	North America	Duck
FJ12025/Muscovy duck/China/2012	Asia	Duck
127/chicken/Netherland/1996	Europe	Chicken
D11-JW-032/Pekin duck/Korea/2011	Asia	Duck
C10-GY-001/chicken/Korea/2010	Asia	Chicken
LS19-3581-1	North America	Chicken
D11-JW-012/Pekin duck/Korea/2011	Asia	Duck
D11-JW-017/Pekin duck/Korea/2011	Asia	Duck
JL-EDSV-629/duck/China/2018	Asia	Duck

2(b) Fiber protein Maximum Parsimony

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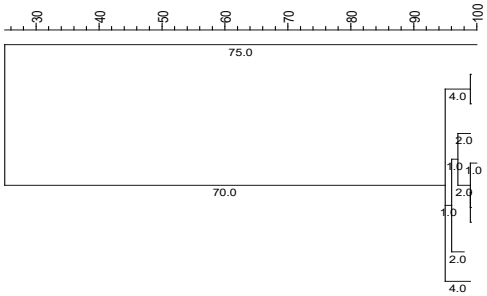
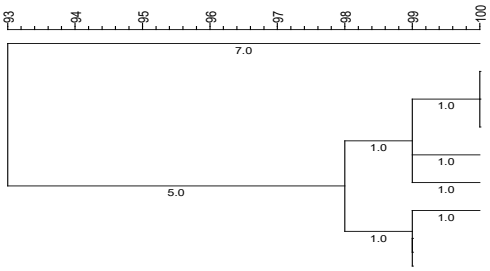


FMV-19-2234581/duck/Canada/2019	North America	Duck
D11-JW-012/Pekin duck/Korea/2011	Asia	Duck
D11-JW-017/Pekin duck/Korea/2011	Asia	Duck
LS19-3581-1	North America	Chicken
127/chicken/Netherland/1996	Europe	Chicken
D11-JW-032/Pekin duck/Korea/2011	Asia	Duck
C10-GY-001/chicken/Korea/2010	Asia	Chicken
FJ12025/Muscovy duck/China/2012	Asia	Duck
JL-EDSV-629/duck/China/2018	Asia	Duck

2(c) Hexon protein Maximum Parsimony

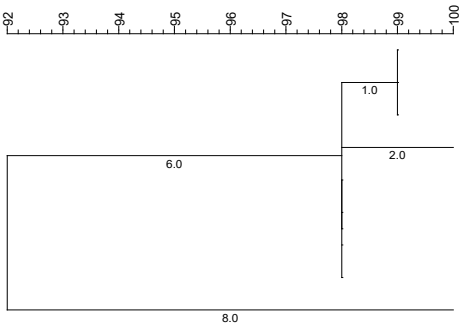
tree from <new cluster analysis>



FMV-19-2234581/duck/Canada/2019	North America	Duck
D11-JW-032/Pekin duck/Korea/2011	Asia	Duck
C10-GY-001/chicken/Korea/2010	Asia	Chicken
127/chicken/Netherland/1996	Europe	Chicken
FJ12025/Muscovy duck/China/2012	Asia	Duck
LS19-3581-1	North America	Chicken
JL-EDSV-629/duck/China/2018	Asia	Duck
D11-JW-012/Pekin duck/Korea/2011	Asia	Duck
D11-JW-017/Pekin duck/Korea/2011	Asia	Duck

2(d) Penton protein Maximum Parsimony

tree from <new cluster analysis>



D11-JW-032/Pekin duck/Korea/2011	Asia	Duck
C10-GY-001/chicken/Korea/2010	Asia	Chicken
127/chicken/Netherland/1996	Europe	Chicken
JL-EDSV-629/duck/China/2018	Asia	Duck
LS19-3581-1	North America	Chicken
FJ12025/Muscovy duck/China/2012	Asia	Duck
D11-JW-012/Pekin duck/Korea/2011	Asia	Duck
D11-JW-017/Pekin duck/Korea/2011	Asia	Duck
FMV-19-2234581/duck/Canada/2019	North America	Duck

FIELD TRIAL COMPARING DIFFERENT VACCINATION PROGRAMS ON BROILER PROGENY PROTECTION AGAINST A PANEL OF VARIANT IBD VIRUSES

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INTRODUCTION

Last year, we reported on the diversity of IBDVs in U.S. broilers surveyed from 2015-19[1]. AL2 viruses made up over half (52%), followed by T1 viruses (17%) and Del-E and Group-6, both at 8%. The remaining 15% fell into a catch-all category of New-types which usually share key mutations of two of the other described groups. Differences between commercial vaccines in cross protection against these various IBDV types have been demonstrated using fractional dose studies[1,2,3]. This paper describes IBDV protection results of progeny broilers coming from breeders on different commercial inactivated IBD programs, with or without the inclusion of an autogenous IBD vaccine.

MATERIALS AND METHODS

Ten flocks of Ross 708 eggs from one broiler company were shipped to the University of Delaware to hatch. All breeder flocks received the same commercial IBD vaccine C in the 10-12 week slot and half of these flocks also received an autogenous IBD vaccine at this handling. For the 5 autogenous vaccinated flocks, 3 received commercial vaccine A and 2 received commercial vaccine B at 18-19 weeks. The commercial vaccine breakdown was the same for the 5 non-autogenous flocks. At hatch, 20 chicks from each of the 10 flocks were co-mingled and placed into each of 6 different colony houses (200 birds per house). At 2 weeks of age, 5 of the colony houses were challenged with 3.0 EID50 IBDV from each of the following groups: Del-E, AL2, T1, Group-6 or New-type; one house remained unchallenged to serve as controls.

The challenge study was terminated at 3 weeks of age when birds and bursas were weighed and clinically reactive bursas were flagged as non-protected. Birds were considered grossly protected if they showed no clinical lesions and their bursa to body weight ratio (B:BW) was higher than the cut-off value of the negative controls (mean B:BW minus 2 standard deviations) of their respective flock. Bursas were placed in formalin containers and scored microscopically for bursal lesions using a 4-point system (1-normal, 2-mild, 3-moderate, 4-severe). Birds were considered protected microscopically if they were free from moderate (1/3 to ½ of follicles involved) to severe lymphoid depletion. A $p \leq 0.05$ level of significance was used for all hypothesis testing with two sided tests.

RESULTS

All but one Autogenous flock (combined with Vaccine A and challenged with Group-6) demonstrated good protection (≥45%) against all five IBDVs (Figure 1) and there were no significant differences in protection between commercial vaccines A and B. However, flocks not receiving Autogenous vaccine (Figure 2) showed a different pattern. Vaccine B program flocks demonstrated protection against all five IBDVs except for one flock on microscopic protection against Group-6. In contrast, two out of three Vaccine A flocks had suboptimal protection against Group-6 and one of these also had suboptimal protection against Del-E (microscopic only), T1 and New-type challenge. In addition, while all flocks lacking autogenous vaccine demonstrated solid AL2 protection, Vaccine B protection was significantly higher (85%) than Vaccine A (67%).

DISCUSSION

This trial demonstrated that two different killed commercial IBD programs were each able to give broad protection against a panel of IBD challenge viruses when supplemented with an autogenous IBD vaccine. However, without the autogenous vaccine, there was a divergence in IBD protection between programs based on commercial vaccine. Namely, the program containing Vaccine B was still able to achieve comparable levels of IBD protection but the program containing Vaccine A saw two of three flocks dip in Group-6 protection and one of these also fell short in T1 and New-type protection. In summary, this

study shows that commercial inactivated vaccines can vary in their ability to cross protect progeny broilers against today's more prevalent variant IBDVs. This should be factored into the design of the entire killed vaccine program including what IBDVs, if any, to include in autogenous vaccine.

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Figure 1. Commerical IBD program protection (with autogenous supplementation)

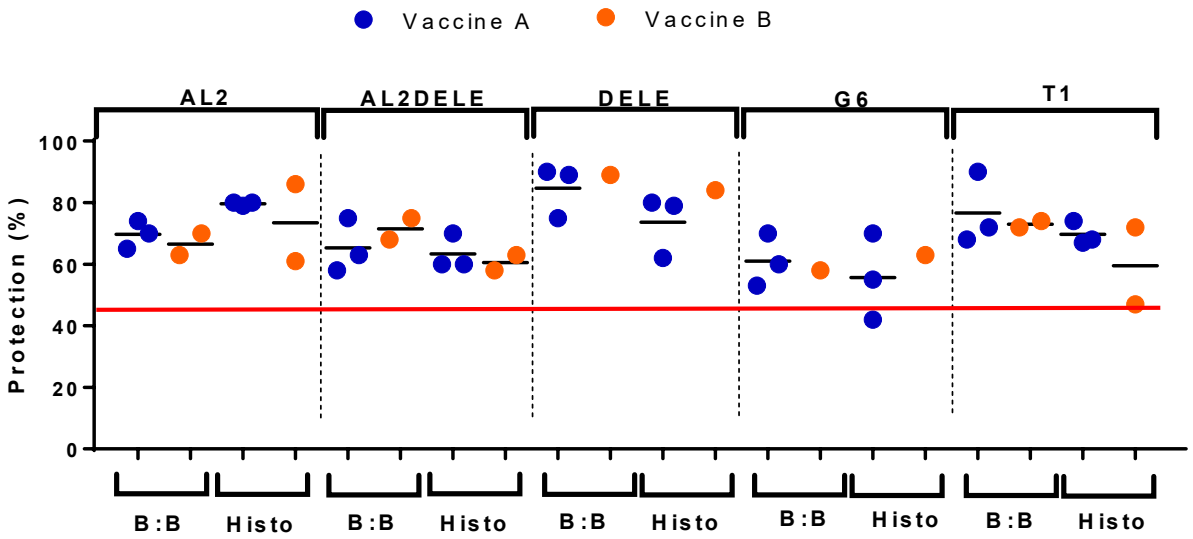
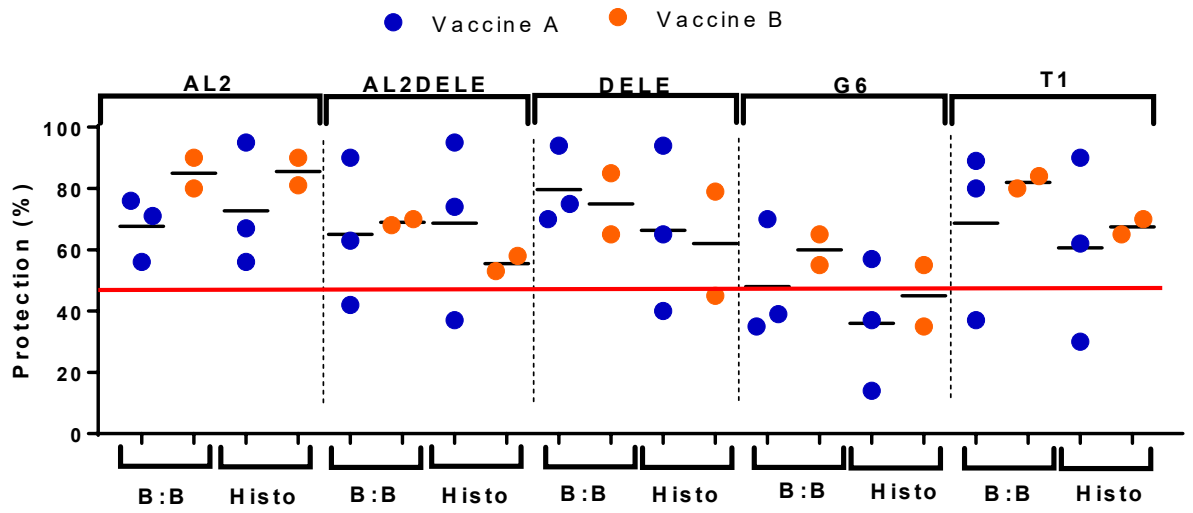


Figure 2. Commercial IBD program protection (no autogenous supplementation)



TI-06111

LIVE *SALMONELLA* TYPHIMURIUM VACCINATION OF TURKEYS RESULTS IN CROSS PROTECTION AGAINST A *SALMONELLA* READING ISOLATE

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INTRODUCTION

Historically the turkey industry has performed extremely well on the Food Safety and Inspection Service (FSIS) *Salmonella* performance standards when evaluated by carcass rinsate samples with approximately 86% (1) of the plants being considered as category 1 (plants are ranked from 1 to 3 based on salmonella prevalence where 1 is the best and 3 the worst categories). Conversely, when the evaluation is performed using ground turkey samples, the majority of the plants are falling into categories 2 and 3 (37 and 44% for categories 2 and 3, respectively). The serotype isolated the most from ground turkey samples is *Salmonella* Reading (1), a serotype that was linked to a major US foodborne illness outbreak between 2017-2019 causing 358 reported cases across 42 states (2). Considering the non-optimal FSIS Salmonella performance from the industry on turkey ground samples and the relative importance of *Salmonella* Reading regarding human illness, the objective of this trial was to assess the efficacy of a live *Salmonella* Typhimurium vaccine (LVST) against a *Salmonella* Reading challenge in turkeys.

MATERIALS AND METHODS

A total of 1,908 day-old male poults were randomly divided among 106 floor pens (18 poults/pen) containing fresh pinewood shavings. There were 12 treatments consisting of a combination of challenge type (age and CFU levels as factors) and LVST status that were randomly assigned to pens in a randomized block design (Table 1).

All the LVST vaccinated poults were spray vaccinated at hatch and received a second dose (boost) of LVST via drinking water at either 21 or 28 days of age. Birds in the challenge pens were given a *Salmonella* Reading inoculum by oral gavage at either 5 days of age (10^6 CFU/bird) or at 39 days of age (10^6 or 10^8 CFU/bird). At day 48, 3 birds per pen were euthanized and had a rinsate with feathers collected for *Salmonella* spp. enumeration (most probable number – MPN – methodology). Each bird was then sampled for cecae (MPN) and liver/spleen (MPN and prevalence) for *Salmonella* spp. evaluation. In addition, one boot swab was collected per pen for *Salmonella* spp. enumeration. Birds were fed a commercial turkey diet meeting the NRC recommendations *ad libitum* using three dietary phases. Turkey body weight and feed were weighed at the end of each dietary phase for performance evaluation (13, 28 and 48 days). All statistical analyses were conducted at a 0.05 level of significance ($P < 0.15$ was considered as a trend) using two-tailed tests.

RESULTS

For the five-day challenge, both enumeration in the cecae and prevalence in the liver/spleen showed low values with no statistical differences associated. For the 39 days challenge, there were no statistical differences observed on the enumeration values in the cecae; however, on the high challenge dose, the LVST with the boost at 4 weeks resulted in 0.6 log numerical reduction when compared with the respective challenge controls. Prevalence and enumeration in liver/spleen samples were significantly reduced by LVST in both challenge doses (Table 2).

No statistical significance was observed on enumeration in bird rinses and bootswabs. However, all LVST treatments numerically reduced *Salmonella* spp. enumeration in bootswabs (reductions from 0.2 to 1.0 log). LVST also numerically reduced *Salmonella* spp. enumeration on birds given the high *Salmonella* Reading challenge dose (three-week boost = 0.5 log reduction; and four-week boost = 0.8 log reduction). There were no statistical differences in bird performance (body weight and feed conversion rate).

DISCUSSION

Vaccination of broilers with LVST has been shown to result in cross protection and load reductions of other important poultry *Salmonella* spp. serotypes including *S.* Enteritidis, *S.* Kentucky, *S.* Heidelberg, and *S.* Infantis (3). The results presented herein showed that LVST vaccination in turkeys has the potential to reduce *S.* Reading overall and limit its spread to the organs. This is especially important

considering the high prevalence of positive samples observed in ground turkey samples from processing plants. It has been suggested that the translocation from the intestines to internal organs has been a concern with ground poultry products (4). Even though there were no statistical differences observed in the cecae *Salmonella* spp. enumeration results, the significantly lower *Salmonella* spp. prevalence and enumeration observed in the LVST vaccinated poults, indicates that vaccination equipped birds with an effective immune response to the *Salmonella* Reading challenge. The effective control of the systemic spreading of *Salmonella* spp. attributable to LVST vaccination has been previously shown for *Salmonella* Infantis challenges in broilers (5). In conclusion, LVST vaccination of turkeys should be considered as a foundational part of a company food safety program considering the industry struggle on meeting the FSIS Salmonella performance standards on turkey ground samples and the deleterious effects of *Salmonella* Reading as a foodborne pathogen.

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Table 1. Treatment factors evaluated and the respective numbers of pens.

Challenge	Vaccination treatment (prime and boost)	Number of pens
Non Challenged	Non Vaccinated	10
Challenged at 5 days of age with 10^6 CFU of *Salmonella* Reading/bird	Non Vaccinated	10
	Live ST (day 0 and boost 3 week)	11
	Live ST (day 0 and boost 4 week)	11
Challenged at 39 days of age with 10^6 CFU of *Salmonella* Reading/bird	Non Vaccinated	10
	Live ST (day 0 and boost 3 week)	11
	Live ST (day 0 and boost 4 week)	11
Challenged at 39 days of age with 10^8 CFU of *Salmonella* Reading/bird	Non Vaccinated	10
	Live ST (day 0 and boost 3 week)	11
	Live ST (day 0 and boost 4 week)	11

Table 2. *Salmonella* spp. prevalence and enumeration in liver/spleen samples for turkeys challenged at 39 days of age.

Challenge	Vaccination treatment (prime and boost)	Prevalence (%)	Enumeration (MPN/g)
Non Challenged	Non Vaccinated	10	0.15
Challenged at 39 days of age with 10^6 CFU of *Salmonella* Reading/bird	Non Vaccinated	60	1.57*
	Live ST (day 0 and boost 3 week)	27*	0.51*
	Live ST (day 0 and boost 4 week)	30**	0.58
Challenged at 39 days of age with 10^8 CFU of *Salmonella* Reading/bird	Non Vaccinated	80	3.16
	Live ST (day 0 and boost 3 week)	55***	1.23*
	Live ST (day 0 and boost 4 week)	70	1.83

*$P \leq 0.05$ **$P \leq 0.08$ ***$P \leq 0.13$

CONCOMITANT VIRUSES ON AVIAN REOVIRUS ISOLATES OBTAINED FROM CLINICAL SAMPLES

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SUMMARY

The selection of pure avian reovirus cultures is extremely important for viral characterizations, antigenicity and pathogenicity studies. Through molecular characterizations, we have detected several isolates in which other viruses have been present i.e. chicken astrovirus, avian rotavirus, adenovirus and chicken parvovirus. In addition, an important number of the screened isolates contained more than one avian reovirus genotype. These findings trouble pathogenicity, antigenicity, and vaccine selection studies and pose the question of the accuracy of those tests and the quality of the autogenous vaccines. Within the avian reovirus isolates that we have analyzed, 91% were positive for one or more of the previously mentioned viruses. Moreover, about 50% of the analyzed samples possessed more than one reovirus genotype. This information adds importance to the screening of avian reovirus isolates before characterization or selection of these isolates for autogenous vaccine production.

INTRODUCTION

Avian reovirus (ARV) is known as the main cause of viral arthritis and tenosynovitis in chickens, causing substantial economic impacts to the poultry industry worldwide. ARV has been associated with respiratory and gastrointestinal infections in addition to immunosuppression. A surge of new ARV variant strains has boosted research in reoviruses. In chickens is not uncommon to find two or more concomitant pathogens in a clinical condition (2,3,6,7,9), sometimes even imbalances on the gut microbiota, including viruses, might cause clinical problems. Moreover, two or more ARV genotypes might be causing co-infections. Tang et al., in 2016 (11) described the presence of two ARVs co-infecting in clinical cases of XXXX in layers. Despite this report, it is not well understood how concomitant viruses and ARV variant co-infections can shape the clinical pictures in chickens. As part of our ARV epidemiological surveillance and pathogenicity studies (1,4), we screened several ARV isolates for the presence of more than one ARV genotype and for concomitant viruses such as astrovirus, rotavirus, adenovirus and parvovirus.

MATERIAL AND METHODS

Samples. Seventy-nine isolates obtained from tendons (65), hearts (6) and intestines (4) were obtained from broiler chickens experiencing tenosynovitis between 2016 and 2018. Isolates were confirmed positive to avian reovirus through RT-PCR.

RNA and DNA viral extraction and detection. RNA was extracted from all isolates using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). DNA was extracted from thirty-two isolates using QIAamp DNA mini kit (Qiagen, Valencia, CA). Viral RNA or DNA detection, from the previously mentioned viruses, was performed using conventional RT-PCR and PCR respectively, according to published protocols (Table 1). Amplicons were visualized using gel electrophoresis.

Whole genome sequencing of ARV isolates. RNA was extracted from 10 ARV isolates. cDNA libraries were prepared using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA). Whole genome sequencing was performed using Illumina HiSeq 3000 at the 100 bp paired end. The viral contigs were determined using NCBI-BLAST with default parameters to find contigs with sequences matching GenBank reovirus sequences.

RESULTS

Concomitant viruses. Ninety-one percent of the isolates analyzed resulted positive to one or more virus. Avian rotavirus was detected in 51 out of 75 isolates (68%), astrovirus was detected in 39 out of 75 isolates (52%), avian adenovirus was detected in 29 out of 32 isolates (91%) and parvovirus was detected in 8 out of 32 isolates (25%). All virus types were found in isolates obtained from tendons and hearts. Astrovirus, rotavirus and adenovirus were found in isolates from intestines (Figure 1).

Co-infection with multiple ARV genotypes. Two or more avian reovirus genotypes were found in six out ten (60%) isolates. The found genotypes were I, IV and VI according to Sigma C.

In conclusion, clinical ARV isolates had one or more concomitant viruses. Moreover, close to half of ARV isolates had more than one ARV genotype. This information adds importance t avian reovirus isolates screening before characterization or selection of these isolates for autogenous vaccine production.

(A full version of this manuscript will be published elsewhere.)

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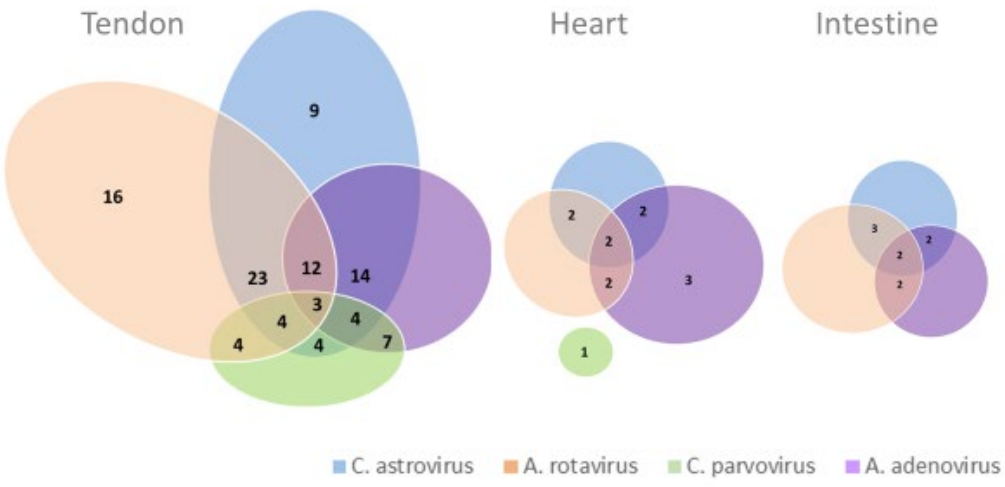
Table 1. Nucleotide sequences of primers used for detection of contaminant viruses.

Virus	Primers	Nucleotide sequence (5′-3′)	Amplicon (bp)	Reference
Chicken astrovirus	CAS pol 1F	GAY CAR CGA ATG CGR AGR TTG	362	Day et al., 2007
	CAS pol 1R	TCA GTG GAA GTG GGK ART CTA C		
Avian rotavirus	NSP4 F30	GCC CGT GCG GAA AGA TGG AGA AC	630	Day et al., 2007
	NSP4 R660	TCG GGT TGG GGT ACC AGG GAT TAA		
Avian adenovirus	MK 89	CCC TCC CAC CGC TTA CCA	421	Xie et al., 1999
	MK 90	CAC GTT GCC CTT ATC TTG C		
Avian parvovirus	PVF1	TTC TAA TAA CGA TAT CAC TCA AGT TTC	561	Zsak et al., 2009
	PVR1	TTT GCG CTT GCG GTG AAG TCT GGC TCG		

Figure 1. Venn diagram showing overlapping concomitant avian viruses in different avian reovirus isolates. Tendon origin isolates: 65, heart origin isolates: 6, intestine origin isolates: 4.



OUTREACH EFFORTS TO PROMOTE AND ESTABLISH GAMEFOWL WELLNESS IN SOUTHERN CALIFORNIA

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SUMMARY

In May 2018, yet another outbreak of virulent Newcastle disease (vND) affected backyard and commercial premises in southern California. To help educate on disease prevention, the UC Davis School of Veterinary Medicine has joined the California Avian Health Education Network (CAHEN) team from the California Department of Food and Agriculture (1) to create open communication networks with gamefowl breeders in the affected areas. By gathering input from gamefowl breeders, both virtually and in-person, the new "Gamefowl Wellness Program" from UC Davis has been able to create educational content in various formats and provide it on several outlets to the public. Nurturing trust with these poultry communities allows us to receive the information needed to develop effective outreach strategies that can better serve them. In responding to their concerns, we can garner their trust that would be necessary to prevent foreign animal disease outbreaks in the future.

INTRODUCTION

The vND virus has periodically caused large outbreaks in California; in 1971-1974, 2002-2003, and most recently in 2018-2020, creating a dire need for educational outreach for backyard poultry enthusiasts. Interestingly, the 2018-2020 vND outbreak affected poultry owners in the same counties of southern California as the previous 2002-2003 oubreak did, further reinforcing the concept that individuals may not have received or be receiving the resources they need to uphold the health of their birds (1). Considering the concentrated numbers of backyard birds that can be present in both suburban and urban areas (2), the 2018-2020 outbreak unfortunately spread from backyard poultry to commercial facilities, affecting 476 premises total. Four commercial premises were infected during this vND outbreak (4), reinforcing the importance of educating poultry owners as means of protecting the commercial poultry industry and consequently, human health.

The answer to approaching poultry communities may be in "personalizing" outreach efforts to fit the needs of each community. For example, while highly pathogenic avian influenza (HPAI) H5N1 has become endemic in Southeast Asian countries, Thailand adapted its HPAI surveillance programs to the poultry systems present in the country and successfully controlled the outbreak in 2008. The Thai cockfighting community were resistant to recommendations and disease reporting requests made by veterinary authorities, leading policymakers to evaluate the structure and implementation of their disease surveillance programs (3). During the 2018-2020 vND outbreak in California, CDFA, the UC Davis School of Veterinary Medicine, and the Western University of Health Sciences collaboratively hosted monthly educational workshops for backyard poultry enthusiasts. Topics like poultry diseases, disease prevention, biosecurity, and vaccination were presented, but workshop attendance was low. Some attendees provided their reasoning for the low attendance: mistrust in governmental authorities.
Like Thai cockfighting communities, flocks at risk in California have demonstrated the same resistance to governmental authorities but have trust-based relationships amongst themselves, important in diseminating information (3). Thus, outreach strategies must be re-evaluated and structured for the specific poultry communities that are being targeted, with the objective of providing them with the resources they need in the format they can use.

MATERIALS AND METHODS

Using the information obtained from the educational workshops and small focus groups of backyard poultry enthusiasts, our objective became to bring the resources directly to these communities through several methods as a new "Gamefowl Wellness Program." To safely reach individuals, we hosted free, virtual workshops open to everyone on online outlets. Workshops were used to present on topics such as vaccination protocols, biosecurity, and disease prevention, and allowed participants to ask

their questions. With the help of the CAHEN program, we networked with prominent individuals within poultry communities, allowing us to reach a broader audience and provide additional information in English and Spanish on avian diseases through podcasts.

To diseminate accurate information, we established a program email address through which gamefowl breeders could reach out to knowledgeable poultry experts with their issues and concerns and get direct answers in either English or Spanish. Through an online survey form, gamefowl breeders could also safely provide their contact information to receive workshop updates and resources. Podcast interviews, informational factsheets, and videos are periodically posted on a UC Davis poultry webpage and advertised through online outlets.

Additionally, instead of holding in-person workshops in locations that were out of reach for these communities, we visited local feed stores frequented by individuals with at-risk flocks and provided Newcastle vaccines to feed store owners. Feed store owners agreed to make the vaccines available to their customers. Vaccines were distributed to feed stores along with factsheets on correct vaccination protocols, procedures, and schedules as a means to establish avian health in the area. Visiting local feed stores provided the opportunity to learn the communities' needs directly from gamefowl breeders, to inform them about the Gamefowl Wellness Program's resources, and more importantly, to foster trusting relationships with the community.

RESULTS

Meeting with gamefowl breeders that were directly affected by the recent vND outbreak, in person and virtually, facilitated our objective of designing outreach efforts for specific poultry communities. Their input allowed us to create the content that is publicly available on the UC Davis poultry webpage and informed us on the topics they wished to learn about. With their advice, we have provided program resources on a social media platform as well, allowing us to reach more gamefowl breeders not only in the outbreak-affected areas but worldwide.

Making not only resources, but communication, between gamefowl breeders and our program more accessible, we have gained crucial input that has greatly developed our outreach strategies. Through the online survey form and email, our team has learned what resources the community needs and has provided answers about avian health for veterinarians in the Southern California area. Local feed store visits have allowed us to communicate information to gamefowl breeders through the feed store owners, which serve as effective intermediates that breeders trust. As constant communicators with breeders, feed store owners have also been strong representatives of poultry communities, informing us of potential concerns. Vaccine takes and population immunity evaluation will be investigated through blood samples from poultry owners that agree to sample their birds

CONCLUSION

Maintaining open communication networks with gamefowl breeders in southern California has helped create the strong trust-based relationships necessary to provide them with useful resources. By maintaining a constant presence in these communities, whether virtual or in-person, we can continue nurturing these trust-based relationships so we can better contribute to their overall avian health. Receiving the input of gamefowl breeders will allow us to improve our outreach strategies and learn how to respond more effectively to their concerns. Over time, these communication networks may help us reduce problems affecting flock health, prevent future disease outbreaks, and more importantly, establish trust with the gamefowl breeder community that can help continue this cycle.

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CHICKEN IGY ANTIBODIES AGAINST SARS-COV-2 PROTEINS: PRODUCTION AND NEUTRALIZATION EFFICACY

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SUMMARY

The novel severe acute respiratory syndrome (SARS)-like coronavirus, SARS-CoV-2, is responsible for the global COVID-19 pandemic. Effective interventions are urgently needed to mitigate the effects of COVID-19 and likely require multiple strategies. Egg-extracted antibody therapies are a low-cost and scalable strategy to protect at-risk individuals from SARS-CoV-2 infection. Laying hens were hyperimmunized against the SARS-CoV-2 S1 protein using three different S1 recombinant proteins and three different doses. Sera and egg yolk were collected at three and six weeks after the second immunization for enzyme-linked immunosorbent assay and plaque reduction neutralization assay to determine antigen-specific antibody titer and neutralizing antibody titer, respectively. In this study we demonstrate that hens hyperimmunized against the SARS-CoV-2 S1 protein produced neutralizing antibodies against SARS-CoV-2. We further demonstrate that antibody production was dependent on the dose and type of antigen administered. Our data suggest that antibodies purified from the egg yolk of hyperimmunized chickens can be used as immunoprophylaxis in humans at risk of exposure to SARS-CoV-2.

INTRODUCTION

Since the emergence of Covid-19 the race to find treatments and vaccines has been constant. Like in infectious bronchitis virus (IBV), a coronavirus that affects chickens, a successful Covid-19 intervention likely requires multiple approaches, and antibody therapies are an attractive strategy to protect at risk individuals from the infection.

Coronavirus entry into cells is mediated by the spike protein, most specifically its S1 portion, a peplomer-like structure anchored to the virus membrane in the form of a trimer. In addition, the S1 subunit of this protein determines virus variability and elicits neutralizing antibodies (1-3). On the globular head of each SARS-CoV-2 S1 protein is a receptor-binding domain (RBD), which specifically recognizes angiotensin-converting enzyme 2 (ACE2) (4).

Researchers are applying the concept of virus neutralization to generate SARS-CoV-2-specific antibodies in animals to be used for passive immunization against the virus in humans. Harvesting antibodies from eggs laid by hens that have been immunized against the spike protein of SARS-CoV-2 is an attractive model to produce protective antibodies due to the scalability, convenience, and low cost (5).

IgY use in human applications is well tolerated and can be administered orally (6, 7). Furthermore, IgY neither binds to human rheumatoid factors nor activates the human complement system (7), therefore reducing the risk of inflammatory reactions as a secondary effect of using antibodies produced in different species. These characteristics make chicken IgY a promising source of new therapies for human viral diseases such as COVID-19 (8) in addition to active immunization i.e. current vaccination strategies.

MATERIALS AND METHODS

Constructs and virus. Both constructs used SARS-CoV-2 S1 protein. The construct used in vaccine C was expressed in HEK 293 cells and was glycosylated while the construct used in vaccines A and B were produced in an expression system and were not glycosylated. Virus neutralization experiments were performed using the 2019-nCoV/USA-WA1/2020 strain sourced from an infected patient in Washington state.

Experimental design. Two hundred 85-week-old laying hens were transported from a table egg layer farm in the California Central Valley to the UC Davis Teaching and Research Animal Care Services facility, where they were placed on pine shavings in climate-controlled BSL-2 rooms. After one week of acclimatization, the hens received two immunizations administered twelve days apart. Vaccines were prepared as an oil-water emulsion with an equal volume of Freund's incomplete adjuvant (Thermo Scientific, IL, USA) using an Ultra-Turrax homogenizer (IKA, Staufen, Germany) at 25,000 rpm

for 10 min. The experimental groups were as follows: vaccine A twice (A/A), vaccine B twice (B/B), vaccine C twice (C/C), vaccine C followed by vaccine B (C/B), and adjuvant only (negative control). Each treatment group was divided into the following subgroups receiving a 2.5-, 5-, or 50-µg dose. All vaccines were administered by intramuscular route in the pectoral muscle in 0.5-mL volumes. Blood was collected from the ulnar vein upon arrival, 21 days following the second immunization, and at the end of the experiment (6 weeks post second dose). Blood was centrifuged and serum was stored at 4 °C for ELISA. One month following the second immunization, eggs were collected every week and stored at 4 °C for IgY extraction. In the sixth week after the last immunization, the hens were humanely euthanized by CO_2 inhalation, immediately followed by cervical dislocation.

RESULTS AND DISCUSSION

Antigen-specific IgY antibodies were detected in the serum of hyperimmunized hens. Commercial laying hens were immunized against the SARS-CoV-2 S1 protein, and blood was collected 21 days following the second vaccination for antigen-specific ELISAs. Birds receiving 50-µg doses from all vaccines demonstrated strong antibody responses, which were higher ($p<0.05$) than negative control titers (Figure 1). At the lower doses (2.5 and 5 µg), only birds receiving vaccine C demonstrated increased antibody responses, which were statistically significant at 2.5 µg.

Antigen A-, B-, and C-specific IgY antibodies were detected in the yolk of hyperimmunized hens. Beginning one month following the second immunization, eggs were collected weekly for ELISAs to detect antigen A-, B-, and C-specific IgY titers extracted from egg yolks. Yolk extracts were diluted 1:400, and specific IgY for each antigen was measured by ELISA using antigen A-, B-, or C-covered plates. Antigen-specific IgY titers extracted from yolks collected 45 days after the second immunization were significant for all tested antigens in birds receiving 50-µg doses, regardless of which vaccine they were administered (Figure 2). Among the birds administered vaccine A/A, egg yolk-derived antibodies were detectable at the lower immunization doses, but the titers were only significant for the 2.5-µg dose. Egg yolk-derived IgY antibodies from birds receiving the lower doses for vaccine B/B were not significantly different from negative control titers. In contrast, egg yolk-derived IgY of hens from the vaccine C/C groups showed significant antibody titers at all doses (Figure 2). The higher antibody levels obtained by antigen C inoculation might be due to the structural differences between antigens. While antigens A and B incorporated the receptor binding domain (RBD), antigen C contained the entire S1. In addition, antigen C was expressed in HEK293 cells compared with a prokariotic system in A and B. HEK293 cells are capable of post transcriptional modifications while prokariotic systems are not. Greater responses mean more antibodies that can be used in preventative applications in humans.

Hyperimmunized hens produced neutralizing antibodies against SARS-CoV-2. Because of the unequivocal IgY responses at all doses, hens immunized with vaccine C/C against the SARS-CoV-2 S1 protein were tested for neutralizing antibodies in sera at three and six weeks after the second immunization and in egg yolk extractions at six weeks post second immunization. Negative controls included IgY from serum and egg yolk of hens that had not been previously vaccinated against infectious bronchitis coronavirus (IBV) of chickens. These birds are tested regularly for the presence of IBV antibodies by ELISA, showing negative results. As expected, the negative controls did not neutralize virus, except for the 1:10 dilution from the egg yolk extraction in which only 76% of virus was detected. IgY from egg yolk neutralized virus up to dilution 1:40, and serum IgY neutralized virus up to the last dilution measured, 1:80. Compared to serum from three weeks post-infection, neutralization was greater in sera obtained at six weeks even though those differences were not statistically significant (Figure 3). SARS-CoV-2 like other coronaviruses are prone to mutations and recombination events. However, residues in the RBD interacting with the angiotensin converting enzyme and cross-reacting antibodies have been found to be conserved (9). Suggesting that prophylactic antibody administration targeted toward the SARS-CoV-2 RBD may retain sufficient neutralizing activity in spite of viral evolution. This situation adds validity to a potential future approach using IgY obtained from eggs of hyperimmunized hens in humans.

(A full version of this manuscript is being prepared to submit for publication in a relevant journal.)

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Figure 1. IgY titers in serum collected 21 days following the second vaccination for groups receiving (A) vaccine A/A, (B) vaccine B/B, and (C) vaccine C/C combinations. Chickens were inoculated twelve days apart, and titers were measured using antigen A-, B-, or C-coated plates. Error bars indicate the mean and standard error. Letters (a-b) indicate significant differences ($p < 0.05$).

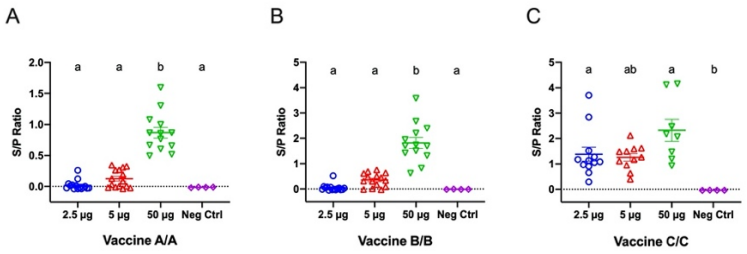


Figure 2. IgY titers from egg yolk collected six weeks following the second vaccination for groups receiving (A) vaccine A/A, (B) vaccine B/B, and (C) vaccine C/C combinations. Chickens were inoculated twelve days apart, and titers were measured using antigen A-, B-, or C-coated plates. Error bars indicate the mean and standard error. Letters (a-c) indicate significant differences ($p < 0.05$).

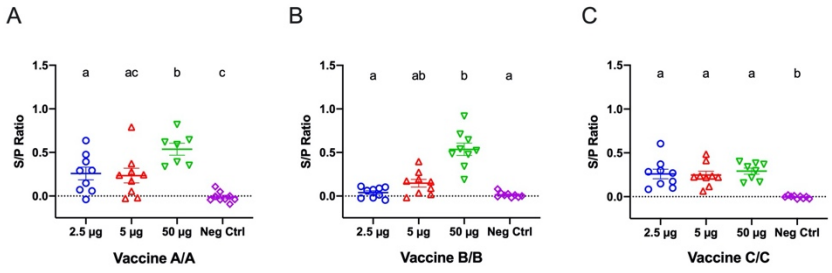
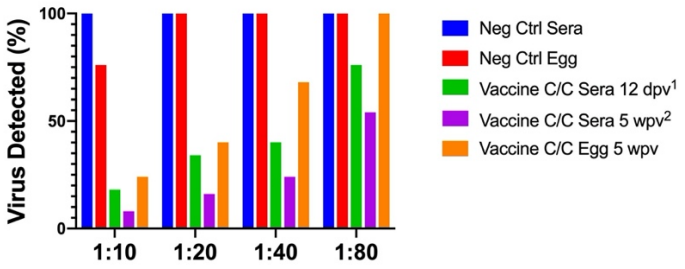


Figure 3. A plaque reduction neutralization assay (PRNA) was performed to detect SARS-CoV-2-specific neutralizing antibodies in sera and egg yolk extractions from hens receiving vaccine C/C or negative control hens with no exposure to IBV vaccine. Viral titer of SARS-CoV-2 was determined by counting the number of plaques and represented as relative percentage of neutralization with respect to the negative control. 1days post-second vaccination, 2weeks post-second vaccination.



WHAT DO WE KNOW ABOUT CORONAVIRUSES IN THE POULTRY INDUSTRY THAT WE CAN APPLY IN SARS-COV-2 RESEARCH?

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SUMMARY

General classification and tropism. Coronaviruses possess a single stranded positive sense RNA genome. This genome is the longest among RNA viruses having between 27 to 30K nucleotides. These viruses are sensitive to disinfectants, heat due to its envelope formed by a phospholipid bilayer which is easily disintegrated by soap. In addition, the capsid, structure that protects the genome, is not tightly sealed like in non-enveloped viruses, making the genome prone to de disintegrated. Finally, all coronaviruses are highly contagious and possess an incubation time between two and 14 days.

The international committee on taxonomy of viruses (ICTV) http://www.talk.ictvonline.org/taxonomy/ classifies infectious bronchitis virus (IBV) as a member of the *Coronaviridae* family in the genus *Gammacoronavirus*, with the beluga whale coronavirus, turkey coronavirus and others. On the other hand, SARS-CoV-2 is classified in the genus *Betacoronavirus* with other coronaviruses affecting humans i.e. SARS, MERS, etc. and other coronaviruses affecting rodents, hedgehogs and bats. Even though these two viruses, IBV and SARS-CoV-2 use similar replication strategies and belong to the same family, they are distant.

Several symptoms have been associated with coronavirus infections in their hosts. While most of coronaviruses show respiratory symptoms (SARS-CoV-2, SARS-CoV, HCoV, FIPV, IBV, BoCoV) or enteric (TGEV, TCoV) outcomes, hepatic (MHV, FIPV) neurologic (MHV, FIPV) and urogenital (IBV) effects have also been reported. In poultry, IBV has been associated with diverse clinical signs due to the effects on different systems (respiratory, urogenital and intestinal). This is due to the tropism that this virus has to epithelial tissue. This tropism in IBV and several coronaviruses is driven by the spike (S) protein and its interactions with receptors in the different tissues. This tropism is different for different coronaviruses.

Origins of SARS-CoV-2. While the closest known CoV to SARS-CoV-2 belongs to a bat with 96% homology, it has been reported closest homologies with CoVs from pangolins. Even though not confirmed, pangolins could be the intermediary host of the SARS-CoV-2. In addition, it has been commented that this virus might have been engineered in a laboratory.

In regards to the sequence homology between SARS-CoV-2 and IBV, the S1 protein on its hypervariable region is between 37 to 45% similar to the S1 protein of SARS-CoV-2. This is considering IBV ArkDPI, Mass and Conn in the comparison. This homology is low considering that differences of 5% between two IBV viruses determine different genotypes. The complete genome of IBV (Beaudette) is 70% similar to SARS-CoV-2. This data doesn't surprise since these two viruses belong to different genotypes. In terms of genetic variation, it has been reported that SARS-CoV-2 changes at a rate of 23 o 24 nucleotides per year compared with IBV that changes at a rate of 40 nucleotides per year. These numbers locate SARS-CoV-2 as less variable than MERS-CoV, HCoVOC43 and IBV that are usually sampled and surveilled extensively.

Receptors in the host. While it is possible that there is more than one receptor for IBV in chickens is of common knowledge that the receptor is salic acid. For SARS-CoV-2 the receptor is the angiotensin converting enzyme 2 (ACE-2). Most of the times the presence of specific receptors interacting with the S1 protein of coronaviruses determine the hosts of these viruses. For IBV, chickens are the host even though IBV has been found in gallinaceous birds without inducing pathology. For SARS-CoV-2 cats, hamsters, ferrets, primates and humans have been found to be susceptible. Dogs, pigs, turkeys, and chickens are not susceptible. As extensively described in IBV the S protein determines geno and phenotypic variability, interacts with cell receptors of the cell determining tissue tropism and elicits neutralizing antibodies; these characteristics makes this protein crucial in generating protective strategies such as vaccines or antibodies. One of the most interesting features of the S gene in SARS-CoV-2 is an insertion of 12 nucleotides in the polybasic amino acid site between S1 and S2. These change makes the virus more prone to cleavage of S1 and S2 making the virus more effective on its replication and infectivity.

Immune responses. Even though, variants are emerging in several parts of the world, only one serotype has been described for this virus compared with IBV that has dozens of serotypes described. This makes prevention by vaccination feasible reason why vaccines are being used in order to generate population immunity and reduce the circulation of the virus. Even though this strategy is helpful, the intense use of vaccination adds selective pressure to CoV. These events add speed to the generation of variants reason why molecular surveillance is extremely important in order to be aware of the circulating strains and take action if new antigenic variants are occurring in a certain area. For IBV mucosal responses and antibodies (IgA and IgY) are crucial in the protection against the disease while antibodies in sera (systemic) are not well correlated with protection. The IBV immunity after disease or elicited by vaccination is not long lived, reason why booster vaccinations are needed. We also know that cellular immunity is crucial, particularly CTLs. Re-infections are common in SARS-CoV patients, this might be due to an elevated innate response blocking or overwhelming the adaptive response. More research is needed in these topics. Newer data reports that Spike and receptor binding domain (RBD) IgG titers in serum were durable with modest decline at six to eight months at population level. The response of the tested individuals is highly heterogeneous being this the key feature for the memory responses. B cell memory responses were present five to eight months after infection and memory T cells six months after infection (Dan *et al*., 2021). These results don't mean that individuals should be protected after infection because the mechanisms of protection have not been elucidated for this virus.

Surveillance. The most important strategy to control or prevent coronavirus infections in a geographic location is constant surveillance. This surveillance needs to use the full genome or segments representing hypervariable regions of the genome. The information gathered should be used to understand the virus evolution and for early detection of antigenic variants. IBV shows a cyclicity of outbreaks between five to seven years. IBV variability is helped by the selective pressure of vaccination (live vaccines). This virus has the capability of staying in chicken populations for a long time helped by the immunosuppression levels in chicken flocks favoring the generation of variant strains.

Vaccination. Vaccination and surveillance are key to control and prevent IBV. In the case of live virus vaccine, the immunity generated is complete and protection should be adequate if homologous types are used for vaccination. The use of vaccine combinations will ameliorate the clinical signs but will not reduce shedding which perpetuates the virus and enhances viral variation in the field. While inactivated vaccines can generate humoral antibodies in high levels (after multiple doses) CoVs usually require local immunity for protection and neutralization. While recombinant vaccines against IBV have been investigated in poultry none of the them have been marketed due to their low efficacy. This might be due to the difficulties of expressing the right neutralizing epitopes or the difficulties of generating local protective immunity.

In conclusion, coronaviruses are a big family of viruses including several animal species and humans. IBV is a gamma coronavirus and SARS-CoV-2 is a beta coronavirus. There are similarities and differences between them. Epidemiology, immunology and molecular biology IBV knowledge can be used to understand the behavior and effects of SARS-CoV-2 in humans. Constant detection and surveillance is necessary to understand circulating coronaviruses and craft good prevention strategies.

STUDYING THE PROTEOME OF NECROTIC ENTERITIS-CAUSING *CLOSTRIDIUM PERFRINGENS* TO IDENTIFY PUTATIVE ANTIGENS

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ABSTRACT

During the past two decades, necrotic enteritis (NE) caused by virulent *Clostridium perfringens* has been extensively studied. No effective control method is currently available, in part due to the lack of a complete understanding of the genetic determinants of NE-causing *C. perfringens* that could contribute to the development of a protective immunity. Six virulent and 10 commensal *C. perfringens* strains were whole genome sequenced and *in silico* analysed. CELLO, Gpos-mPLoc and FUEL-mLoc softwares were used for protein localisation prediction, while TMHMM and SignalP softwares identified transmembrane regions and signal peptides. The immunogenic potential of each protein was predicted using VaxiJen®. Virulent *C. perfringens* presented 9.3% more proteins than the commensals. Virulent strains also revealed 14.8% more probable antigens, with nearly 20% of these showing an immunogenic score above 0.9. Finally, virulent *C. perfringens* showed 36.8%, 7.6% and 0.7% more proteins identified as extracellular, cytoplasmic and membrane-bound, respectively, than the commensals.

INTRODUCTION

A reduction in the use of antibiotics by the poultry industry has been linked to the re-emergence of avian necrotic enteritis caused by virulent *Clostridium perfringens* worldwide (1). This has prompted the scientific community to better understand the pathogenesis of NE through research work including studies on *C. perfringens* virulence. Even if many alternatives to antibiotics have been proposed, to date, no effective control method is available and a vaccine strategy would represent one of the most attractive methods to control NE for the poultry industry (2). During the past few years, different potential *C. perfringens* antigens have been evaluated for their role in the development of a protective immunity in broiler chickens and up to now, none has been considered for the commercialization of a fully protective vaccine against NE (3-8). This highlights the need for a complete understanding of the genetic features of NE-causing *C. perfringens* that could contribute to the development of a protective immunity.

MATERIALS AND METHODS

Strain selection. Six virulent and 10 commensal *C. perfringens* strains isolated from both healthy and NE-affected broiler chickens were selected based on the results from previous studies (9, 10).

Genomic data. Libraries were prepared with the Nextera™ XT DNA Library Prep kit (Illumina procedures guide, Doc #15031942). The MiSeq Sequencer System Software (Illumina) was used to filter raw sequence reads. Assembly was performed (INNUca assembly pipeline (https://github.com/B-UMMI/INNUca), followed by annotation (RASTtk annotation tool (http://tutorial.theseed.org/services/docs/invocation/Iris/iris.html)) and genome quality assessment (QUAST quality assessment tool ((http://quast.sourceforge.net/)).

Protein localisation and immunogenic score. Four softwares were used to document bacterial protein localisation: pSORTb (https://www.psort.org/psortb/), CELLO (http://cello.life.nctu.edu.tw/), Gpos-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/Gpos-multi/) and FUEL-mLoc (http://bioinfo.eie.polyu.edu.hk/FUEL-mLoc/). For each protein analyzed, a consensus decision-making approach was used to assign the final localisation. When two distinct predicted localisations were equally suspected, both results were considered. TMHMM® (http://www.cbs.dtu.dk/services/TMHMM/) and

SignalP® (http://www.cbs.dtu.dk/services/SignalP/) softwares predicted the presence of transmembrane regions and signal peptides, respectively. When no signal peptide was identified, the SecretomeP online software (http://www.cbs.dtu.dk/services/SecretomeP/) assessed the presence of an alternate secretion pathway. The online software VaxiJen® completed each protein profile by attributing an immunogenic score to each one of them. Protein scores were categorized from weekly antigenic (0.5-0.7), moderately antigenic (0.7-0.9), antigenic (0.9-1.5) and highly antigenic (>1.5)

RESULTS

In total, 155 700 distinct proteins were identified from the *C. perfringens* genomes analyzed in this study. For both virulent and commensal *C. perfringens*, cytoplasmic proteins were more abundant, followed by membrane proteins and by extracellular proteins that were present in lower numbers, as general statistics showed that 13%, 65% and 22% of the proteins identified could be classified as extracellular, cytoplasmic and as membrane proteins, respectively (see Table I). Statistical analysis showed that virulent *C. perfringens* genomes comprised significantly more proteins coding sequences than their commensal counterpart ($p < 0.0181$), with the extracellular ($p < 0.0001$) and cytoplasmic ($p < 0.0382$) putative proteins identified in higher numbers in the virulent strains analyzed. When using the VaxiJen software, virulent *C. perfringens* revealed both a significantly higher number of probable antigens ($p < 0.0001$) and higher number of antigenic proteins [0.5 and 0.7 ($p < 0.018$), 0.9 and 1.5 ($p < 0.0025$)].

CONCLUSION

In the perspective of fully relying on antibiotic replacement strategies for the control of NE in commercial broiler chicken flocks, results from the current study further emphasize the importance of better describing NE-causing *C. perfringens* genetics. Evaluating the role of unique genetic markers is essential to understand virulent *C. perfringens* strains in both the disease pathogenesis and in the development of a protective immunity in exposed birds.

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(The full-length article will be published in BMC *Bioinformatics*.)

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Table 1. Statistics referring to the virulent and commensal *C. perfringens* strains analyzed.

Statistics	Strain type	
	Virulent	Commensal
	Average	
Protein content		
Total no. of proteins	3390	3099
-Extracellular	487	356
-Cytoplasmic	2187	2032
-Membrane-bound	716	711
Immunogenic potential		
% of the genome identified as antigenic	33.87%	32.27%
No. of potential antigens	1148	1000
No. of weekly antigenic proteins	896	808
No. of moderately antigenic proteins	196	212
No. of antigenic proteins	55	40
No. of highly antigenic proteins	2	0.1

NECROTIC ENTERITIS IN IMMUNOSUPPRESSED BROILER CHICKENS EXHIBITS A MORE SEVERE DISEASE FOOTPRINT

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ABSTRACT

Necrotic enteritis (NE) is a multifactorial disease of broiler chickens caused by *Clostridium perfringens* (CP) type G. Several predisposing factors, including diet, immune status, and stress, affect NE disease progression. The present study examined NE predisposing factors, immunosuppression, and sudden diet change, to evaluate their impact on the disease severity. Experimental broiler chickens (Ross-308) were divided into six groups (n=20/group), [1= normal control, 2= CP in feed (20% protein), 3= CP in feed (28% protein), 4= variant-IBDV (vIBDV) + CP in feed (20% protein), 5= vIBDV + CP in feed (28% protein), 6= vIBDV + CP via oral gavage (28% protein in feed)]. Groups 4, 5, and 6 were immunosuppressed by vIBDV at 17 days of age (3 days before exposure to CP). All chickens were euthanized at 24 days of age. The mortality pattern, gross and histopathological lesions, revealed a more severe NE disease pathology in immunosuppressed groups.

INTRODUCTION

NE is an economically significant disease of broiler chickens caused by CP, a gram-positive, rod-shaped, spore-forming, and anaerobic bacterium. *C. perfringens* affects broiler chickens between 2-6 weeks of age (2). The mortality can reach 1% per day with total mortality of 10-40% (6). Clinical signs such as depression, diarrhea, dehydration, ruffled feathers, low body weight and increased feed conversion ratio (FCR) are typical with NE (3).

Necrotic enteritis is affected by several factors, including nutritional, management, and immunosuppressive mediators. Immunosuppression caused by pathogens like vIBDV and coccidiosis predisposes chickens to NE (8). The frequent incidences of NE have been reported in birds infected with IBDV and those administered with high doses of an IBDV vaccine (7).

Infectious bursal disease virus targets specifically the bursa of Fabricius, the site for the B-lymphocytes development in chickens. The IBDV rapidly replicates in B-lymphocytes, causing rapid necrosis and depletion of B-lymphocytes from the bursal follicles (5), (4). IBDV-mediated destruction of B-lymphocytes abrogates humoral immunity, leading to immunosuppression.

Given a high prevalence rate of variant IBDV in the Saskatchewan broiler chicken industry (about 43% of farms or (52% of barns) (9), we designed the study to investigate the contribution of vIBDV-induced immunosuppression in the incidence of NE.

MATERIALS AND METHODS

Day-old Ross broiler chickens (n=120) were randomly allocated into six experimental groups each consisting of 20 birds/group [1= normal control, 2= CP in feed (20% protein), 3= CP in feed (28% protein), 4= variant-IBDV (vIBDV) + CP in feed (20% protein), 5= vIBDV + CP in feed (28% protein), 6= vIBDV + CP via oral gavage (28% protein in feed)]. Groups 4, 5, and 6 were immunosuppressed by vIBDV at 17 days of age (3 days before exposure to CP). Broiler chickens were fed antibiotic free chicken starter with 20% protein for 18 days, followed by an abrupt increase to 28% protein. Classical clinical signs associated with NE such as depression, anorexia, ruffled feathers, brown foamy diarrhea and deceased mobility were observed in all CP exposed groups.

The challenge strain of CP had α-toxin, netB, cpb2, and TpeL toxin genes. CP was grown in cooked meat medium (Sigma Aldrich) for 24 h at 37 °C under anaerobic conditions. Cooked meat medium was added to the thioglycollate medium (Sigma-Aldrich) at 3% (v/v). Cooked meat medium culture was incubated anaerobically at 37 °C for 15 h. The bacterial growth at 15 h culture had approximately 1×10^9 CFU/mL. The thioglycollate broth-grown culture was then mixed with feed at a ratio of 1:1 (v/w). Feed was withdrawn prior to exposure of birds to CP.CP was administered twice daily either in feed or orally for 3 consecutive days. Birds were observed for clinical signs and mortality post-challenge, and the experiment terminated 5 days after the bacterial challenge. The intestinal tract of all the birds were examined for macroscopic lesions of NE and histopathological lesions.

RESULTS AND DISCUSSION

No mortality was observed in the control group, while mortality was observed at day three post-challenge in groups exposed to CP. Total mortality in group 4 given vIBDV + CP in feed (20% protein) and group 6 given vIBDV + CP via oral gavage (28% protein in feed) were 15% and 10% respectively. Gross pathological lesions typical of NE were observed throughout the small intestine. Birds exposed to CP had classical histopathological lesions such as severe diffuse necrosis of villi associated with bacterial colonization along with infiltration of minimal inflammatory cells. Histopathological lesions in-group 4 given vIBDV + CP in the feed (20% protein) were 45-50% higher than the control group 2 given CP in the feed (20% protein) only. Similarly, histopathological lesions in-group 5 given vIBDV+ CP in the feed (28% protein) were 20-30% higher than the control group 3 given CP in the feed (28% protein) only.

We have repeatedly reproduced the NE animal model in the commercial broiler chickens in a laboratory setting. In our study, the mortality pattern, gross and histopathological lesions were consistent with field cases of NE in broiler chickens. We are using this animal model in our further studies to explore the pathogenesis of CP in commercial broiler chickens and ongoing work of developing a vaccine against NE.

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WEB-CRAWLING OF SOCIAL MEDIA TO ANALYZE BACKYARD POULTRY OWNERS RESPONSES TO THE 2018-2020 VND OUTBREAK IN SOUTHERN CALIFORNIA

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SUMMARY

As social media becomes an ever-increasing staple of everyday life and a growing percentage of people turn to community driven platforms as a primary source of information, the data created from these posts, articles and connections can provide a new source of information from which to better understand an event in near real-time. The 2018-2020 outbreak of virulent Newcastle Disease (vND) in Southern California is the third outbreak of vND in California within a 50-year time span. These outbreaks are thought to be primarily driven by the density of backyard poultry flocks in the region, illegal bird fighting and the likely illegal movement of birds into the U.S. from south of the U.S. border. The 2018-2020 outbreak was the first outbreak of vND that was widely discussed online by bird owners, and anti-depopulation groups alike. Here we employed a "web crawling" tool to collect all available online mentions of virulent Newcastle Disease (vND). Initial data analysis has shown a peak of social media posts in March and April of 2019 which preceded the formation of the anti-depopulation group Save Our Birds (SOB). Analysis of social media showed that Twitter was the most significant source of comments over blogs, forums and other news sites. Further analysis of these posts for positive or negative sentiment showed a majority of negative posts with a peak during May of 2019. We believe that the utilization of web-crawling and web-scraping tools are a novel and important tool in outbreak mitigation strategies. The results of this study can be used to improve time to regulatory response and reduce the number of animals affected by vND and other disease outbreaks in the future.

INTRODUCTION

vND is a highly contagious and lethal disease of poultry caused by avian paramyxovirus 1 (APMV-1). While vND is endemic to many parts of the world, it is not considered endemic to the United States (1). Recent outbreaks of vND in Southern California in 1971-1973, 2002-2003, and most recently 2018-2020 have all required extensive culling of millions of birds and caused significant economic damage to the poultry industry (2). Past vND outbreaks in the region began in game fowl illegally imported for fighting and exhibition (3), which are especially prevalent in Los Angeles, Riverside and San Bernardino counties, all at the center of the 2018-2020 outbreak. Often taking place at private residences, bird fighting and exhibitions offer a financial incentive that result in the frequent movement of birds to different properties, even when quarantines are in place. This potential for disease spread is compounded by the large number of backyard poultry owners in the region and in some cases their proximity to commercial poultry operations. The illegal nature of bird fighting in the U.S. as well as the large number and wide distribution of poultry owners in southern California has historically posed a challenge to understanding bird movements during outbreaks and the public perception of the governmental response.

As the internet becomes firmly ingrained as a staple of daily life, it also becomes an increasingly larger catalogue of publicly available data that people choose to provide about themselves. In 2019, 90% of all adults in the US reported using the internet (4), and 72% of them reported using at least one social media platform (5). These data are currently used by various companies to understand purchasing and other behaviors (6). In the past, Google Flu Trends (2009-2015) has attempted to harness search engine data as an epidemiological predictor of flu outbreaks with varying results. A later analysis of the project revealed that Google's methods can be used to reduce errors in CDC models by up to 52.7% compared to CDC data alone (7). The ability to use these data to better understand perception, behavior and knowledge associated with an epizootic event is not understood to the best of our knowledge. The aim of this study is to investigate correlations between the timeline of the CDFA regulatory response to the 2018-2020 vND outbreak and social media conversations about the outbreak as well as understand perception of depopulation efforts and other outbreak mitigation strategies through sentiment analysis. Since this is the first outbreak of virulent Newcastle disease during the

height of the social media age, these insights can serve to better inform outreach efforts during future outbreaks.

MATERIALS AND METHODS

Data collection: Social media related to vND was collected using an enterprise web-crawling service. A standard Boolean string search was set up initially searching for posts including both English and Spanish in the United States and Central America from 30 June 2018 to 22 July 2020. The following Boolean string was used in the search:

(((chicken OR rooster OR "hen" OR "Hen") NOT pox)
AND
("virulent Newcastle Disease" OR (Newcastle NEAR/20 disease) OR "vND outbreak"
OR NDV OR vND OR vNDV))
NOT
(flu OR influenza OR COVID-19 OR corona OR coronavirus OR COVID OR Sars-CoV-2)

A similar search was performed in Spanish. The Boolean string search returned all available mentions that include chicken, rooster or hen and some variation of vND or Newcastle disease. The search also specified the exclusion of chicken pox, influenza, and all terms relating to COVID-19.

Trends in the social media data were compared to the significant events in the outbreak as identified by the CDFA website[7]. These points were compared to the post volume, lead or lag time compared to the CDFA announcement, location (where available), platform and sentiment. Sentiment analysis was performed with tools built into the web-crawling platform.

RESULTS

The 2018-2020 vND outbreak in California was first detected by the California Animal Health and Food Safety (CAHFS) Laboratory in May of 2018, but the web-crawling service employed has a backlog limit of two years. Therefore, the first two months of the outbreak were not captured using the web-crawling service. A total of 2,498 posts from 862 unique authors were returned using the Boolean string search. Searches with the same or equivalent terms in Spanish returned less than 150 relevant results, and the majority of those were from news reports. For these reasons, Spanish language postings were not included in this study. Initial analysis of data collected without specifically excluding mentions of chicken pox, influenza, and COVID-19 resulted in a very noisy dataset with false peaks due to the COVID-19 pandemic. After further data cleaning, 1,764 relevant posts were used for analysis. Figure 1 shows the volume of online conversation over time with peaks in the collected data marked "A" through "F."

Peak "F" is followed by a sustained increase in post volume compared to the two-year median weekly mention volume (11 mentions per week). This peak does coincide with the first detected cases in Riverside County, and the majority of the posts are local news stories and retweets of a tweet linking an article on the outbreak. This peak does see the first negative sentiment reaction to mandatory depopulation reported by the *Press Enterprise*, a local newspaper in the Inland Empire. Peak "C" is primarily driven by several retweets, including one by the American Veterinary Medical Association, of an article posted by all Southern California News Group (SCNG) Newspaper Twitter accounts titled, "Chicken-killing Newcastle disease prompts widespread quarantines in Southern California."[10] Retweets of this news story continued into peak "B", and were followed shortly after by another SCNG-wide news story "Chicken-killing Newcastle disease prompts euthanasia orders for parts of Chino." The words "Chicken-killing" and "euthanasia" caused negative overall sentiment during these peak weeks. Peak "A" is primarily driven by retweets of an article by *The Counter* titled, "Backyard chickens hit hard by a long-gone, extremely contagious disease." The first news reports and tweets of backyard bird owners taking issue with euthanasia efforts by CDFA occur during peak "D," with words and phrases including "protest," "chicken slaughter," "beg," and "killing everyone's birds" driving a sharp spike in negative sentiment. Peak "E" was driven by an article about designer chicken coops with a reference to vND published by several Tribune Publishing Company newspapers. This peak is also the first time during the vND outbreak that sentiment was significantly more positive than negative.

DISCUSSION

The results of this study demonstrate that valuable insight on the public's understanding of an outbreak event and its perception of outbreak-mitigating action by regulatory agencies can be gained from monitoring relevant social media posts. Throughout the outbreak, posts were made to chicken and poultry specific forums weekly asking questions about the vND outbreak and informing other poultry owners of updates from CDFA and the state veterinarian. These posts tended not to lean extremely negatively towards positive or negative sentiment, but

we're often focused on preventative measures to guard against infection and mandatory depopulation. This result suggests that information is being effectively disseminated to backyard stakeholders. However, these niche forums, like backyardchickens.com (684,000 visits per month) have relatively little reach to the general public compared to large social media platforms like Twitter (1.4 billion visits per month). Even individual Twitter accounts, e.g. The Press-Enterprise, can have over 100,000 followers that view what they post. Posts and reposts by these large social media accounts have the capacity to significantly influence public perception outbreaks and actions taken by governmental agencies to combat them. Several months of Tweets that displayed a largely negative sentiment towards the outbreak and how it was being handled preceded the formation of the Save our Birds Facebook group, increasing anti-depopulation rhetoric, and more widespread cases as bird owners' broke quarantines.

Enterprise web-crawling tools such as the one used in this study act as a good basis for identifying platforms to monitor and generalized analysis. However, some significant limitations were noted including the web-crawlers limited ability to perform sentiment analysis and its inability to track classified postings including Craigslist and Facebook Marketplace. Further targeted web-crawling and using purpose built Natural Language Processing (NLP) packages in R or Python for "beyond polarity" sentiment analysis may provide additional insight.

CONCLUSION

The age of social media requires not only that outbreaks be met with effective response and clear communication, but also that the general public is in favor of the methods used. Using web-crawling tools to monitor social media for content related to specific disease outbreaks and analyzing the sentiment of these posts is a novel and effective method of engaging with stakeholders during disease outbreaks. The methods described above allow for improved response to subjects that are ambiguous or incorrectly understood by the general public, and they allow for direct social media response to posts that address mitigation efforts negatively.

(The full-length article will be submitted to the journal *Transboundary and Emerging Diseases*.)

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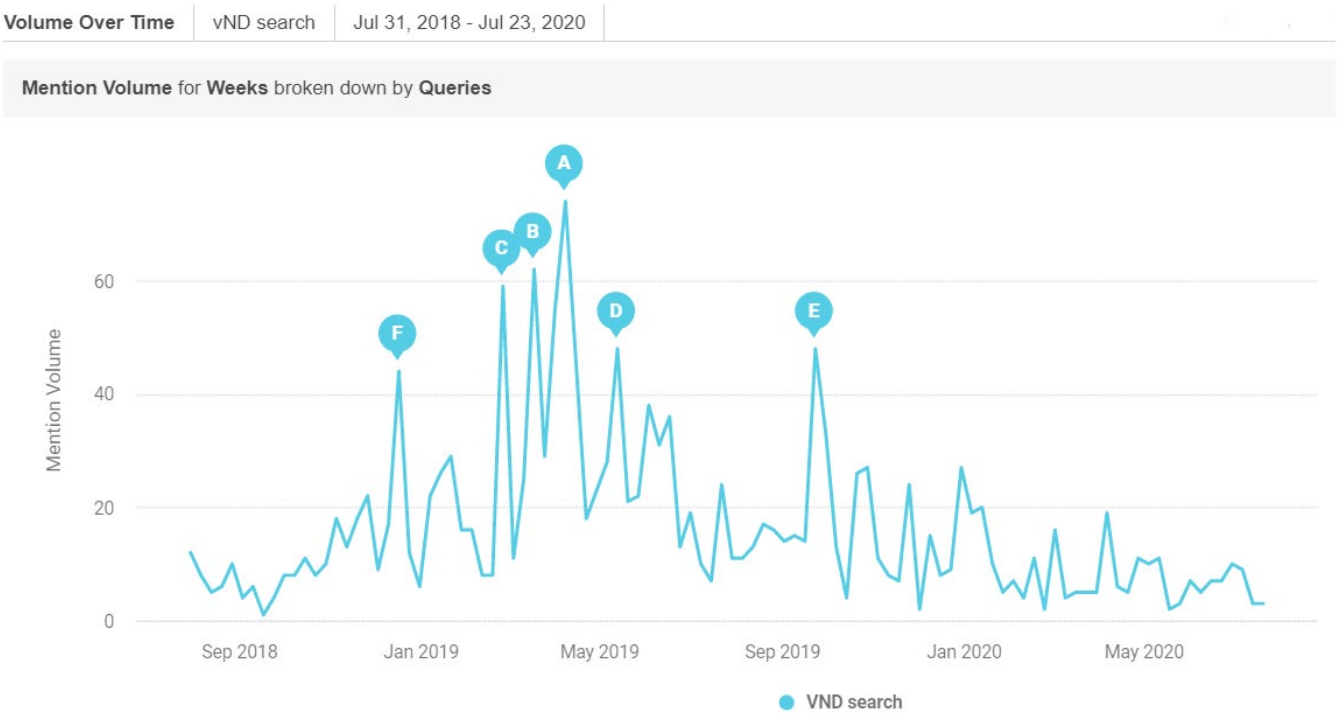
Figure 1. Volume of on-line conversations related to vND between July 31st 2018 and July 23rd 2020.



HOW WAS THE US EGG INDUSTRY AFFECTED BY THE EVENTS OF 2020?

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SUMMARY

The events of 2020, mainly the effect of the COVID-19 pandemic, caused disruption of normal business in regard to sales to the food service industry, some problems with absenteeism due to illness of employees, and investment in labor and supplies to increase sanitation efforts. Those operations serving the foodservice industry were negatively affected while those serving the retail industry benefited.

INTRODUCTION

The pandemic caused by the coronavirus named COVID-19 began to infect Americans in late January of 2020. State governments began calling for closing of schools and restaurants and for restricted travel. Business travel declined due to fear of COVID-19 infection which affected sales for hotels and restaurants. Travel for pleasure was also dramatically curtailed.

If an employee of an egg company, hatchery, or crew became a victim of COVID-19, the person would need to be out at least 10 days plus any persons in close contact with the infected person would need to quarantine at home for 10 days.

As with the meat plants where employees are carpooling and work in close proximity to one another, adoption of preventative measures such as partitions between work stations and break room seating, mask wearing requirements, hand sanitizer station addition, increased labor and materials for sanitation, decreased capacity of break rooms to provide more separation, staggered break times, etc. were done.

Several egg industry people either called or were sent a set of questions to answer in regard to the effect of the pandemic on their operations and formed the basis of this presentation.

DISCUSSION ON THE EFFECTS OF THE PANDEMIC

The four major effects from the pandemic on the egg industry was the reduction in foodservice sales, employee illness effects, increased expenses to keep employees healthy, and the effect on suppliers.

Reduction of foodservice sales. The foodservice industry started to decline down in March of 2020 with a total reduction of foodservice sales of 36% in March. This reduced level of sales continued through the summer and began to increase somewhat in late 2020 with some increase in restaurant openings.

Retail sales of eggs skyrocketed due to people now eating their eggs at home. Many grocery shelves were empty due to panic buying. Egg prices also rose to quite high levels (see the accompanying figure 1) due to the high demand for retail shell eggs. Prices then declined once the industry adapted to the increase retail demand and there was an excess of eggs from the breakers available on the market. Liquid egg markets that mostly serve the foodservice industry were very depressed. Some frozen eggs that could not be sold by their expiration date could not be given away and went to the landfill.

Those operations that were not selling to the foodservice industry such as the organic and other specialty eggs have never had better prices throughout the pandemic. People who used to eat eggs at restaurants now were eating eggs at home and were willing to pay for the higher priced specialty eggs.

The increase in cartoned egg sales resulted in a shortage of the desired cartons. Generic cartons had to be used so advertising on cartons was reduced. Carton prices have also increased due to higher demand.

Transportation costs for shipping eggs to foodservice have increased due to the lack of being able to fill trailers to capacity as before. Also, truckers are not allowed to unload their own trucks due to COVID-19 restrictions at the destination.

Several million layers were depopulated earlier than normal due to the lack of demand for their eggs.

Employee illness due to COVID-19. This problem did not affect the egg industry nearly as seriously as it did the meat processing industry.

A portion of egg processing plant workers, hatchery workers, chicken house caretakers, moving and vaccination crew members, owners, flock supervisors, etc. were infected and required quarantine at home. To my knowledge, no egg processing plants were closed or had to divert production to another processing facility due to COVID-19.

Employee absenteeism resulted in attempts to hire temporary help or add duties on to those workers who were not infected. Luckily, the labor pool had increased as other industries laid off people hence more people were applying for jobs.

Efforts were made to discourage carpooling as this is a real risk factor in the spread of COVID-19. Some operations provided transportation to aid in this effort.

Increased expenses to keep employees healthy. Most all producers had to increase expenditures for masks, thermometers, hand sanitizer, and other PPE items due to the pandemic.

Time was taken to monitor employee's temperatures prior to entry.

Break rooms were rearranged to allow increased distancing while on breaks. Break times were staggered when possible to compensate for the decreased capacity of the break rooms. Some added partitions between employees, air filtration, and extra ventilation to the break rooms. Prior to the pandemic, three companies installed hydrogen peroxide generation systems to sanitize the air in the processing rooms for reducing Salmonella risk which would also reduce airborne coronavirus.

Partitions between workstations in the processing plants were installed by some companies.

Two companies supply their employees with an immune system enhancing supplement to increase their resistance to pathogens.

Much more time and more supplies for sanitation have been required to assure that the premises are virus free for the next shift.

Effect on suppliers. Construction was hindered due to delays in supply of materials, especially electrical supplies.

Lumber prices escalated. For example, a new house will cost 10% more now compared to pre-pandemic.

Trucking costs have risen due to a shortage of drivers.

Animal health companies were unable to send their representatives to visit customers for in-person visits to restaurants, offices, regional meetings, or national meetings due to travel restriction policies. Phone calls and video conferences became popular but were reported to not be as valuable as in-person meetings.

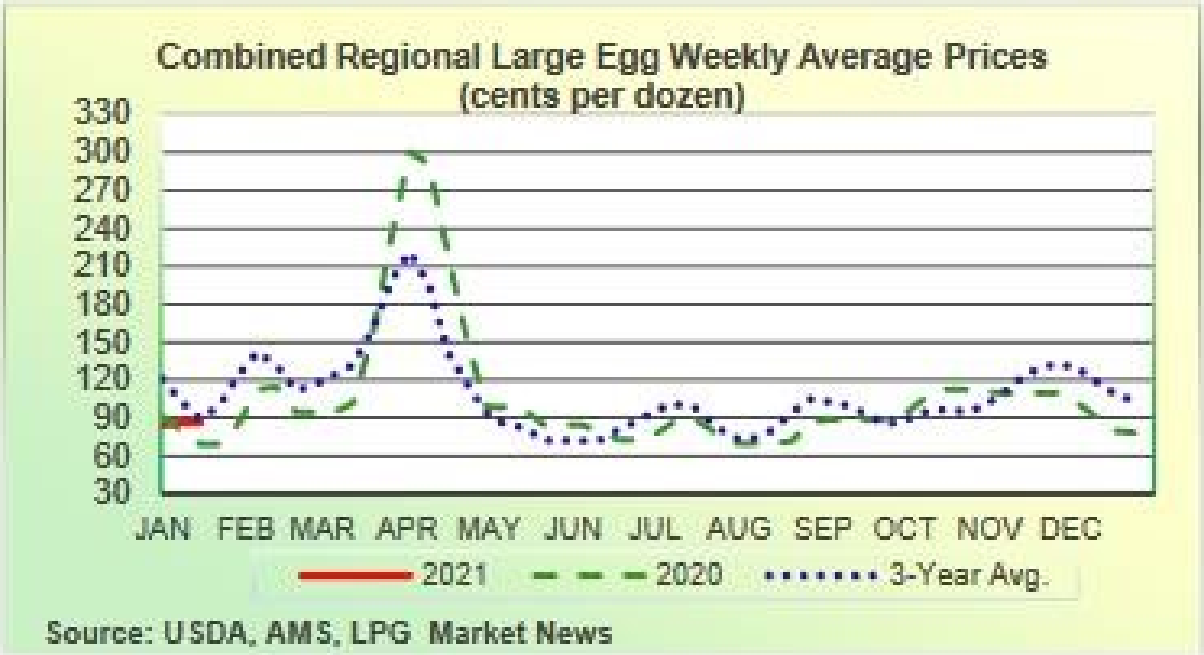
Veterinary consultations were also significantly curtailed due to travel restriction policies. FaceTime or similar video methods were used frequently to view chicken house conditions or necropsy lesions.

FUTURE IMPACT OF 2020 ON THE EGG INDUSTRY

The long-term effects on egg pricing will depend on how long the reduction in restaurant and other foodservice egg consumption will continue to cause low egg prices. This will depend on how soon most people can become immunized and the foodservice business can resume to normalcy.

Electronic technology use will likely continue by veterinarians for providing pullet and layer health services as this was found to be a very convenient, biosecure, and timely method of providing service without traveling to the premise.

Figure 1. Egg Price History, 15 Jan 2021



DEVELOPMENT AND EVALUATION OF RECOMBINANT AVIAN PARAMYXOVIRUS SEROTYPE 10 VECTOR VACCINE AGAINST HIGHLY PATHOGENIC H5 AVIAN INFLUENZA

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SUMMARY

The H5 subtype of highly pathogenic avian influenza (HPAI) viruses pose a serious threat to the poultry industry worldwide. By the use of reverse genetics, we constructed a recombinant avian paramyxovirus serotype 10 vector vaccine expressing the hemagglutinin protein of AI virus H5 subtype (rA10-H5) from the North American influenza outbreak. To evaluate the induction of neutralizing antibodies and efficacy of rA10-H5, white leghorn chickens were vaccinated at 3 weeks of age, boosted 9 days later, and challenged at 7 weeks of age with H5 HPAI viruses belonging to different clades. To monitor viral shedding, oral and cloacal swabs were collected on days 2, 4, and 7 after challenge. The experimental vaccine demonstrated clinical protection and a significant decrease in viral shedding from challenged birds when compared to the control groups. Further studies are needed to fully determine the coverage against possible exposure of other HPAI H5 strains.

INTRODUCTION

Avian influenza (AI) is a highly contagious disease that is caused by infection with influenza A viruses, categorized into high or low pathogenic influenza viruses according to its pathogenicity in chickens (1). Influenza A viruses originating from wild birds are subtyped by reactivity to antisera against two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) to H1-16 and N1-9, respectively (2). High pathogenicity avian influenza (HPAI) viruses are limited to either the H5 or H7 subtypes and possess multiple basic amino acid residues at the HA proteolytic cleavage site, leading to an extremely contagious, multi-organ systemic disease of poultry with high mortality (3). The H5N1 HPAI virus emerged in China during 1996 and has subsequently evolved into diverse clades and subclades (4). Beginning in early 2014, a distinct group of HPAI H5 reassortant viruses (H5N8 clade 2.3.4.4) began to cause outbreaks in poultry in South Korea, and by late 2014, it had spread to China, Japan, and Europe, with multiple isolations occurring from wild birds, even including seemingly healthy birds (5). These Eurasian (EA)-origin H5N8 avian influenza viruses were first detected in North America in December 2014, following the largest recorded foreign animal disease outbreak experienced in NA, with over 48 million commercial poultry dying or euthanized from exposure to an H5 HPAI virus (6, 7). This devastating impact on commercial poultry renewed interest in developing intervention strategies, including vaccines against HPAI viruses.

An experimental recombinant Newcastle disease virus (NDV) and several other avian paramyxoviruses (APMVs) vaccines with an avian influenza HA gene insert showed the potential for use as vaccine vectors (8). However, there is concern that routine vaccination against NDV in commercial chickens, maternal antibodies, or antibodies occurring from natural infection could reduce vaccine efficacy due to their antigenic cross-reaction with recombinant vaccines. In contrast, APMV-10 seems to be exclusive to only their natural host – penguins (9-12). Also, the results obtained in earlier findings show the susceptibility of APMV serotypes to replicate in chickens with no clinical signs (13).

In this study, we generated and evaluated a recombinant APMV-10-based vaccine expressing the HA gene with the typical cleavage site sequence of a low pathogenicity avian influenza (LPAI) virus, that was altered from a polybasic cleavage site of the H5N8 HPAI virus strain isolated in NA in 2014. Variable protection in chickens from experimental challenges with the 2014 NA H5N8, 2014 EA H5N6, and 1995 Mexican (Mex) H5N2 HPAI viruses was demonstrated.

MATERIALS AND METHODS

Viruses. All viruses used in this study were obtained from the Southeast Poultry Research Laboratory's (SEPRL) repository of the United States National Poultry Research Center (USNPRC). APMV-10 strain penguin/Falkland Islands/324/2007

and HPAI strain A/Gyrfalcon/Washington/41088-6/2014 (H5N8 from NA outbreak) were used for the recombinant vector vaccine construction. Three HPAI challenge viruses were used to evaluate the efficacy of the created vector vaccine rA10-H5: A/Gyrfalcon/WA/41088-6/2014 (NA H5N8), A/Chicken/Vietnam/NCVD-14-A324/2014 (EA H5N6), and A/Chicken/Queretaro/14588-19/1995 (Mex H5N2).

Rescue of recombinant virus. Rescue of the rA10-H5 virus was performed by transfecting the full-length cDNA clone pA10-H5 and supporting plasmids into HEp-2 cells as previously described (14-16). The rescued virus was amplified by inoculating of the infected cell lysate into the allantoic cavity of 9-day-old specific pathogen free (SPF) embryonating chicken eggs (ECEs) and incubating the embryos at 37 °C. After 4 days of incubation, the allantoic fluid was harvested and the presence of the rescued virus was detected by the hemagglutination (HA) assay (17). HA-positive allantoic fluid was diluted in phosphate-buffered saline and amplified in chicken embryos three times. The full genome sequence of the rA10-H5 virus was determined by next-generation sequencing as previously described (18, 19).

Biological characterization of rA10-H5. Titers of the rA10-H5 virus were analyzed by the 50% egg infective doses assay (EID_{50}) in 9-day-old SPF chicken embryos (17). Pathogenicity of the recombinant virus was determined using the standard mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays according to the World Organisation for Animal Health recommendations (20, 21).

Vaccination and challenge experiment. Three-week-old SPF white leghorn chickens (*Gallus gallus domesticus*) were divided into 6 groups (n = 10 per group, two groups per virus). Each bird in groups 2, 4, and 6 were vaccinated with 10^6 EID_{50}/0.1ml of rA10-H5 at 3 weeks of age and boosted 9 days later with the same dose. At 19 days post-vaccination (DPV), blood from all the birds was collected to evaluate the humoral antibody response by hemagglutination inhibition (HI) assay. At 20 DPV, the birds in groups 1 and 2 were challenged with a homologous NA HPAI virus, groups 3 and 4 – a heterologous EA HPAI virus, and groups 5 and 6 – a Mex HPAI virus. All birds were challenged with 10^6 EID_{50}/0.1ml of HPAI viruses through the oculonasal route. The challenged birds were monitored daily for clinical signs of AI disease for 14 days. At 2, 4, and 7 days post-challenge (DPC), oropharyngeal (OP) and cloacal (CL) swabs were collected from each bird for detection of challenge virus shedding by quantitative real-time RT-PCR via amplification of the AI virus matrix (M) gene as previously described (22). After 14 DPC, the remaining birds were bled and humanely euthanized.

RESULTS

Insertion of the AIV HA gene with a modified cleavage site compatible with the LPAI virus into the APMV-10 backbone resulted in a slight pathogenicity increase of the recombinant virus, as evidenced by an increased ICPI in day-old chicks. This increase in pathogenicity is small and the vectored vaccine is still considered non-virulent. The MDT of rA10-H5 remained the same compare to the parental APMV-10 and was greater than 168 hours for both. Additionally, no significant difference in the viral yields, measured by EID_{50}, were detected between rA10-H5 and the parental virus, indicating that insertion of the attenuated AIV HA gene in the P-M intergenic region of APMV-10 did not significantly affect replication of the recombinant virus.

After the initial vaccination and the boost with the recombinant virus, the chickens appeared healthy without any signs of vaccine side-effects. After challenge with the HPAI viruses, the birds were examined daily and clinical signs were scored. From 2 DPC, most birds in the control groups exhibited typical clinical signs of the disease. While the challenge with the NA HPAI virus resulted in 80% mortality in the control group, both EA and Mex viruses killed all birds in the control groups by 3 and 5 DPC, respectively.

The recombinant A10-H5 vaccine provided 100% protection in the SPF chickens when challenged with the homologous NA HPAI virus. On the contrary, the recombinant vaccine could not fully protect against the EA and Mex viruses challenge, resulting in 90% and 70% mortality, respectively. Still, the viral shedding data showed significantly higher protection against the NA and EA HPAI viruses, which was indicated by a significant reduction of the viral excretions through the OP and CL routes in these vaccinated groups compared to the control groups, as well as delayed MDT. All surviving birds were checked for seroconversion after challenged with the HPAI viruses.

CONCLUSION

In summary, in the present study, we successfully generated an APMV-10-based recombinant virus that encodes the attenuated HA gene of the H5 HPAI virus that emerged in NA in 2014. We have shown that insertion of the HA gene did not significantly affect replication of the recombinant virus. The pathogenicity tests demonstrated the safety of the rA10-H5 virus in day-

old chickens, and that it can be used for immunization tests in bird studies. The SPF birds vaccinated with this recombinant virus were fully protected against a lethal challenge with the antigenically homologous NA HPAI virus and partially protected against challenge with the heterologous EA and Mex HPAI viruses. Compared with the control groups, the recombinant vaccine induced a stronger immune response in birds, indicated by reduced clinical signs, viral shedding, and a delayed MDT after challenge. The results suggest that the APMV-10 may be a promising recombinant vaccine vector candidate against HPAI viruses, yet needs further development to better match vaccine antigen to field viruses.

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MACROCOCCUS CASEOLYTICUS – NEWLY EMERGING PATHOGEN OF LAYERS CAUSING CRANIOFACIAL INFECTIONS AND LOWERED PRODUCTION

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SUMMARY

Micrococcus caseolyticus, formerly designated as *Staphylococcus caseolyticus*, is a common colonizer of animals and animal-product production sites. Whole genome sequencing of *M. caseolyticus* has demonstrated the capacity to host virulence genes as compared to the established pathogen *Staphylococcus aureus*. Few reports have established *M. caseolyticus* as a pathogen and its potential to cause disease, while most have resolved its participation within the larger microbiome at these animal production environments. In this report, *M. caseolyticus* was isolated from the site of infection in layers experiencing mortality, reduced egg production, and craniofacial discharge. *M. caseolyticus* was also isolated from the cloaca of birds not experiencing disease. Inputs relevant for layer production were also positive for hosting *M. caseolyticus* including incoming pullets to the production house and feed samples. Genetic relatedness of isolates determined those isolated from birds in the cloaca were closely related to pathogenic isolates at the site of infection. Together, *M. caseolyticus* is an emerging pathogen that may be more prevalent in production systems than previously reported.

INTRODUCTION

Micrococcus caseolyticus, a Gram-positive bacterium, is commonly associated with several agriculture environments, but only recently has attention for being an animal pathogen (1). Originally designated as *Staphylococcus caseolyticus*, *M. caseolyticus* was updated to its current genera after re-evaluation of the 16s rRNA sequence (2,3). Characterization of the *M. caseolyticus* genome demonstrated capacity for the bacteria to contain several virulence genes that are established for success in infections (4). Further studies have confirmed that the bacterium can cause infections in animal models through primary inoculation routes (1). Apart from model infection studies, several reports indicate the capacity for *M. caseolyticus* to be a resident within sites of animals or the environment (5,6). Therefore, further understanding of the route of infection and co-occurring circumstances need to be elucidated to add to the pathogenic biology of the organism.

Infections in layers is not always obvious because sequela may only be demonstrated through small changes in production, behavior, or mortality. More overt effects of disease may include stunting, paleness, paralysis, and lethargy. *Staphylococcus* infections, and now *Micrococcus* infections, can present with more dramatic external changes that are characterized with swelling of extremities, caseous exudate, or other discharge from the site of infection (7). Interestingly, as a separation between the two types of disease presentations, staphylococcal infections are more commonly associated with hock and joint infections, while reports of *M. caseolyticus* have included craniofacial targeting (1,8). Most staphylococcal infections are still associated with other health challenges in the bird or requires a portal of entry (9). For *M. caseolyticus* much is still unknown about the general infectious process or the cycling behavior of the bacteria that can propagate infections over house of birds.

Available molecular genetics tools to classify bacteria are enhancing field diagnostics and help to determine important details on pathogens. Initial screen plating on agar plates that are complexed with field observation data have traditionally provided findings relevant to classical pathogens with their treatment. However, newly emerging bacteria and pathogens may have similar findings on characterizable agar plating but would mislead to the ultimate identification (10). As new molecular genetic markers of disease or pathogens are revealed, a new approach to bacterial control can be created to support not only treatment needs but also risk management.

In this study we characterize the presentation of *M. caseolyticus* infection from a layer farm in the Mid-west. Birds displaying disease were between 35 to 55 weeks of age. Of the eleven houses on the farm, all houses contained some diseased birds so one house was chosen to study as a representation of the production system. Primary presentation of disease

showed caseous exudate, swelling of the cranial region and mucus within upper trachea. Infected layers produced fewer eggs and were at significant risk of mortality. What was unknown at the time of initial survey of disease was the sources of introduction of *Macrococcus* to production environments and transmission patterns of *Macrococcus* in a house. Together, we characterized initial introduction of from reservoirs and the relationship of strains between birds and the environment within the house.

MATERIALS AND METHODS

Sampling and specimen handling. Sterile swabs were used to swab surfaces of diseased exudate, tissue, and the cloaca of birds. Pit manure samples were collected into sterile whirl-pak bags using a sterile scope for 1x use. Immediately after collection, samples were maintained at 4°C and processed within 24 hours of collection. Swabs were equilibrated in 10% peptone broth and mixed thoroughly to release bacteria into the media. Subsampling of media. Pit manure samples (50g) were also equilibrated in peptone broth and processed through a stomacher thoroughly mix the sample. A portion of the liquid was removed and utilized for further analysis.

Culture and enumeration of bacterial isolates. From mixed media of swab samples, liquid was applied onto agar plates via spiral plater and quantified through standard photometric analysis (11,12). *E. coli* was processed onto MacConkey-Sorbitol agar for selective and differential plating under standard atmospheric conditions at 37°C. *Clostridium perfringens* was processed onto ChromoSelect agar under anaerobic conditions and incubated at 37°C. *M. caseolyticus* was processed onto Mannitol-Salt agar and incubated at standard atmospheric conditions at 37°C. Colonies on MSA were picked and processed for sequencing of 16s rRNA to determine actual designation of isolate (see below for 16s method).

16s population analysis. From the swab tip used to collect a sample, the swab tip was removed and placed directly into a well of a 96 well bead beating plate included in the Qiagen MagAttract Powersoil DNA Kit. Manufacturer's instructions were followed resulting in DNA extraction of total metagenomic DNA from each swab sample. Once metagenomic DNA is purified it is prepared for 16S community sequencing as follows; DNA is diluted 1:5 by adding 20 uL of molecular biology grade water to 5ul of purified DNA at 0.1 – 10ng/uL. 2 uL of the diluted DNA is then added to a PCR reaction along with 25 uL of ABI Universal TaqMan Reaction mix without UNG (ThermoFisher #4326614), 0.1uL each PCR primers at 100 uM (Illumina-V4-515F-RJ: TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGGTGCCAGCMGCCGCGGTAA, Illumina-V4-806R-RJ: GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGGGACTACHVGGGTWTCTAAT) and 24.8 uL of Molecular Biology Grade water for a total volume of 50 uL. Reactions are thermal cycled as follows; 10 min at 95°C followed by 35 cycles of 95°C 15sec + 55°C 30sec + 72°C for 2 min. Amplified reactions are purified using Ampure XP Magnetic Beads (Beckman Coulter A63881) as per manufacturer's instructions. 2 uL of each amplicon pool is then indexed in a second PCR reaction using the same conditions as above with Illumina XT Index Primers (Illumina XT v2.0 #FC-131-2001-2004) for 15 cycles. Indexed amplicons are then pooled and purified with AmPure XP Magnetic Beads. Pooled, indexed, amplicons are quantitated using the Kapa Illumina Library Quantification Kit (KAPA #KK4835) as per manufacturer's instructions. Purified, quantitated, indexed, pools are loaded on the Illumina MiSeq at a final concentration of 9 pM along with 10% Illumina PhiX (Illumina FC-110-3001). Sequencing is run for 2 x 250 Paired End cycles and the FASTQ files are used to generate relative abundance data.

Random amplification of polymorphic DNA (RAPD). gDNA was extracted from isolates of interest using the Macherey-Nagel NucleoSpin 96 Tissue kit (#740741.4). RAPD analysis was carried out against the 10-mer primer Primer2 (5'-GTTTCGCTCC-3'), using standardized RAPD beads (GE Healthcare #259100-96). The thermocycler method used was 95°C for 5 minutes, followed by 45 cycles of 95°C for 1 minute, 36°C for 1 minute, and 72°C for 2 minutes (with up-ramp rates of ~0.86°C/sec and down-ramp rates of ~2.35°C/sec), followed by 72°C for 7 minutes. The resultant products were resolved into a banding pattern via capillary gel electrophoresis, using a Qiagen QIAxcel Advanced system. From the banding patterns, phylogeny trees were generated in BioNumerics, using Dice at 1% optimization and 1% tolerance to calculate similarity coefficients, and UPGMA to construct clusters.

RESULTS

To investigate the risk of disease from a bacterial pathogen, a sequence of sampling events was organized to provide identification of an enriched pathogen at the site of infection and to characterize reservoirs of the pathogen in the production system (Fig. 1). As a field diagnostic approach, Koch's postulated were lent to the model to first identify a pathogen that is in higher density at site of infection compared to reservoirs (13). Secondly, an organized sampling pattern was used to correlate bacteria in cases of disease and carriage within healthy

population. Finally, evidence of transmission was investigated to demonstrate the bacteria are linked to pathogenic disease. The conclusion of the data should support a relationship map of isolates to show both the connection of isolates from several sites, while also aiding improved biosecurity practices.

Initial investigation of birds displaying disease (yellow cranial exudate, swollen head, lethargic movements) were sampled using a swab applicator. Disease samples were taken directly from the exogenous exudate. Samples from five different birds within the same house all demonstrated a highly enriched population of *M. caseolyticus* (Fig 2.). Interestingly, samples were able to show colonies on Mannitol-Salt Agar (MSA) but were corrected for the classification based on sequencing of the 16s rRNA. Cloaca samples were taken from the broader population of birds that that were not showing signs of disease. Most the most birds were negative (5/20 positive) for *M. caseolyticus*. Other bacteria from cloaca samples were also investigated because of their link as indicators of health including *E. coli* and *C. perfringens*. Both bacteria were compared to the internal DuPont library of sampling data and were found to be within limits of routine densities found in birds that are matching ages. Isolates of *E. coli* and *C. perfringens* were tested for presence of virulence genes and were found to have minimal capacity for virulence.

M. caseolyticus has been commonly found with environmental-associated bacterial populations within an animal production environment; however, little is known as to its introduction in poultry environments. There are no publications indicating the initial introduction of *M. caseolyticus* to layers. To determine introduction routes for *M. caseolyticus*, inputs to layer production including incoming pullets, manure, feed, and the environment were tested as reservoirs (Fig 3). Of the nine incoming pullets tested coming off the truck, two tested positive for *M. caseolyticus* after swabbing their cloaca. Manure from the truck delivering pullets was also positive. Three separate feed mill samples before delivery at the farm and three feed samples from hoppers at the farm were tested. Both feed and hoppers were positive for *M. caseolyticus*, but the overall density of the bacteria was low compared to cloaca or manure samples. Of the panel of sites tested, other environmental locations were negative including the cage, feed bins, feed chain, egg belt, and walkway through house (Table 1).

Microbiome analysis supports the understanding of not only colonizing bacteria, but also bacteria that may cause disruptions to the relationships within the microbiome (14). Six birds from the house that contained birds displaying active disease were sampled from both their cloaca and from the manure pit. These birds were not displaying disease at the time of sampling. Of the over 400 different bacteria elucidated from 16s sequencing profiling, *M. caseolyticus* was identified to be within the thirty most abundant bacterial genera (Fig 4.). *M. caseolyticus* had an average relative abundance of 6.95% within the total population in the cloaca, while only a relative abundance average of 0.1% in manure. A high abundance of unclassified bacteria from both the cloaca and manure were found when comparing sequences to the curated database. Several other common bacteria to poultry production were identified. Specific pathogens to poultry were not identified within cloaca or manure other than the *Macrococcus* including genera: *Clostridium* pathogenic spp., *Pasteurella*, *Campylobacter*, *Salmonella*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*.

Of the total isolates found from birds, disease, and environmental sampling a RAPD analysis scheme was used to determine percent relatedness (Fig 5.). Wet swabs were specific to cloaca sampling of birds within the house that displayed active disease. Diseased tissues swabs were taken from exudate of birds showing disease. Manure samples were from separate pit samples from under the house. The RAPD dendrogram displayed thirteen of the disease tissue samples were grouped to those isolates found from wet swabs (cloaca). Four disease samples grouped with manure samples. Average percent relatedness for manure to disease samples was 88.1%, while percent relatedness for cloaca to disease samples was 97.7%. Most isolates had some relationship to larger group of isolates, while a small number of isolates were had no relationship.

DISCUSSION

M. caseolyticus has been found in several types of animal production environments and has recently been isolated from poultry production environments. Although not abundant, more articles are identifying *M. caseolyticus* as a pathogen to birds (1,4). Due to the potential of *M. caseolyticus* to also culture similarly to pathogenic staphylococci, many previous *M. caseolyticus* infections may been classified at *Staphylococcus* infections. The updated classification of *Macrococcus* from *Staphylococcus* may indicate *M. caseolyticus* infections are more wide-spread than previously understood.

Previous *M. caseolyticus* infections in birds describe symptoms related to infected craniofacial regions and discharge. What was poorly understood was routes of infection or reservoirs that support the bacteria within the production environment. After identifying *M. caseolyticus* from sites of infection,

further analysis showed the bacteria was a resident within the population of birds in the house. Other pathogens did not appear to be increased or decreased in relation to Macrococcus colonization. Although not pursued within this study, viruses may also be supporting this type of infection (15). Other publications have not supported the theory of *M. caseolyticus* as a secondary pathogen, but it remains to be resolved (1).

Inoculation of birds from *M. caseolyticus* may occur through environmental associations because of isolation of the bacteria from production environment. However, inputs to layer-type production were not monitored for *Macrococcus* colonization or carriage. This included testing and isolation from young pullets, feed, mechanical-associated parts, or water. Pullets, not associated with the infected house, did show positive cultures for *M. caseolyticus*. Pullets remain a key area of focus for future research as to when these birds are colonized in their life before brought into production houses. Feed was also positive for the bacteria. It was hypothesized before testing that casein in feed may be associated with the bacteria. Casein is processed in the feed mill that is responsible for feed sent to the production location tested. Further testing will be required to determine if certain feed components support *M. caseolyticus* growth.

Key findings from microbial population studies showed *M. caseolyticus* can be a resident within the microbiome of the cloaca of layers and is also a more minor component of manure microbiome (2,5). Establishment within the microbiome may be the key factor in maintenance of the bacteria within the production system and propagating infection (16). Further comparison of the isolates also showed consistent relationships to those found within the cloaca, more so than comparing to manure isolates. This finding helps to show pathogenic cycling may be propagated through bird-to-bird associations.

Because of the relationship of pathogenic isolates to birds, biosecurity was updated to the production environment. Birds displaying disease or are suspected of early disease through temperature increases, lethargy, or swelling are to be removed. The farm responsible for raising the pullets is currently being screened to understand carrying capacity for the bacteria and potential health management options before birds are transported to the production farms. Manure and cage surfaces are also being supervised more closely when performing disinfection procedures. Finally, feed disinfection is being investigated with the use of commercially-available products for their success against *M. caseolyticus*.

To provide support of the microbiome, an in-feed probiotic is currently being used to help in improving health of the birds. This probiotic is a novel combination of five strains of *Bacillus*. Two of the five strains of *Bacillus* can produce secondary metabolites (bacteriocins) to support health of the microbiome, while the other three strains produce exogenous proteases. This product has been tested for its improvement not only in bird health, but also in supporting the manure environment for composting and improved physical qualities.

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Figure 1. Sampling scheme for layer group. This scheme is to describe the flow of questions and sampling for the approach to studying M. caseolyticus in layers. Final applications in management and targete probiotic use are the desired outcomes from the cumination of data.

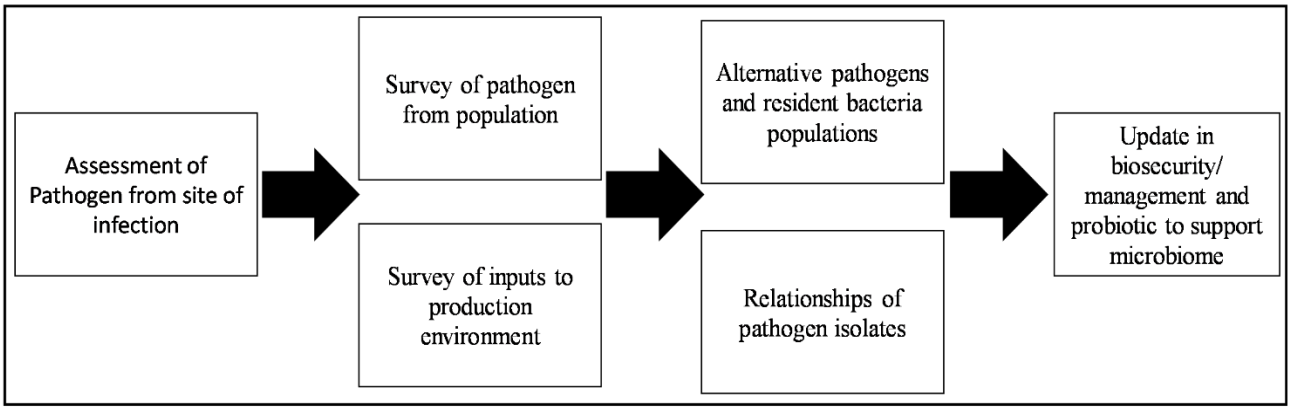


Figure 2. Isolation and characterization of M. caseolyticus in house. (A) Five separate birds displaying active disease with craniofacial discharge sampled in triplicate, mean and SD calculated for samples per bird. (B) Survey of cloca sampling of birds within the house displaying active disease breaks. Bacterial density quantified after overnight growth. (C) E. coli and C. perfringens from cloaca. Samples taken in triplicate with mean and SD.

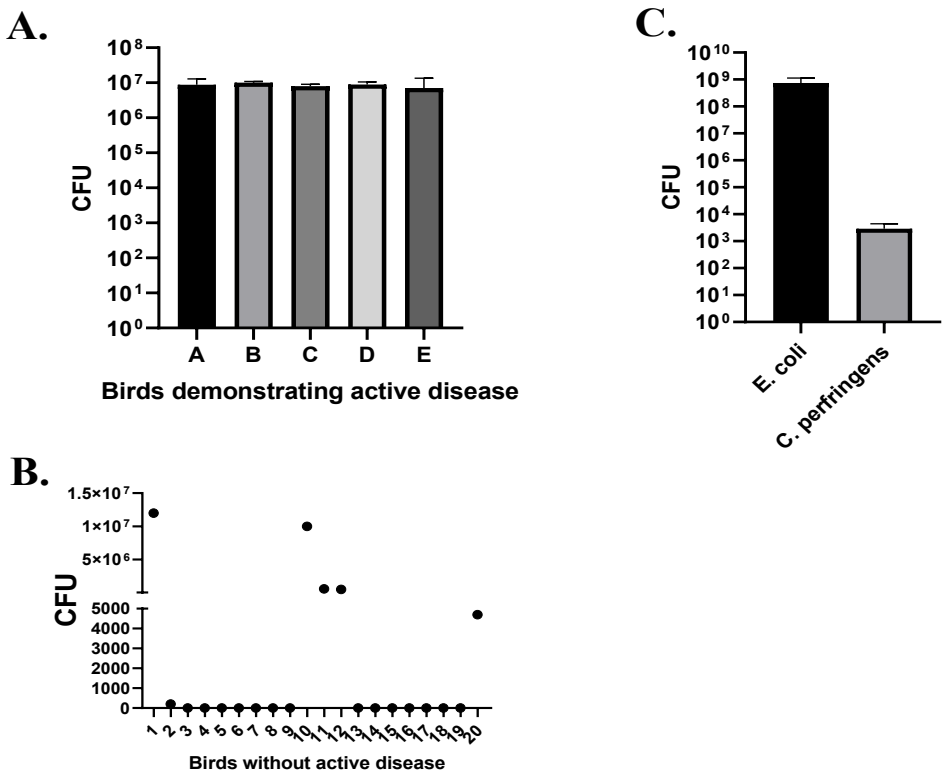


Figure 3. Screen of layer inputs for *M. caseolyticus*. Samples positive for *M. caseolyticus* in screenlisted with individual density data and combined for average (bar) and SD (errors).

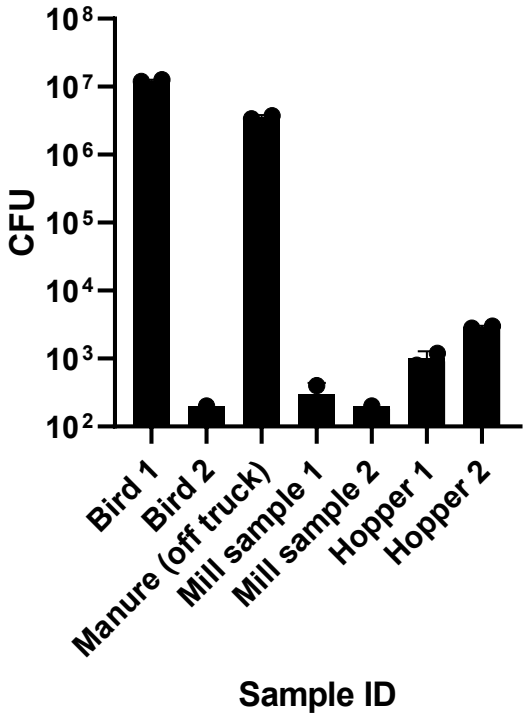
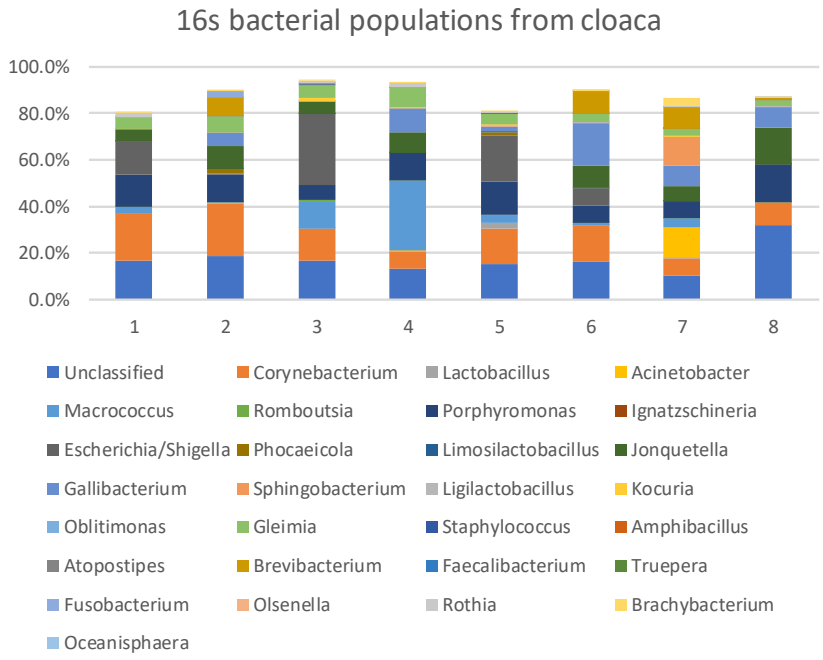


Figure 4. Total population of cloaca and manure from layers in house displaying disease. Eight seperat birds and nine separate manure analyzed for total population of bacteria through 16s rRNA sequencing. Relative abundance of reads through sequencing compiled as total relative abundance and then individual bacteria listed with color for abundance. Height of bar indicates cumulativeralative abundance of bacteria. The 29 bacteria with highest relative abundance listed in the graph with 400 different bacteria species identified.

A.



B.

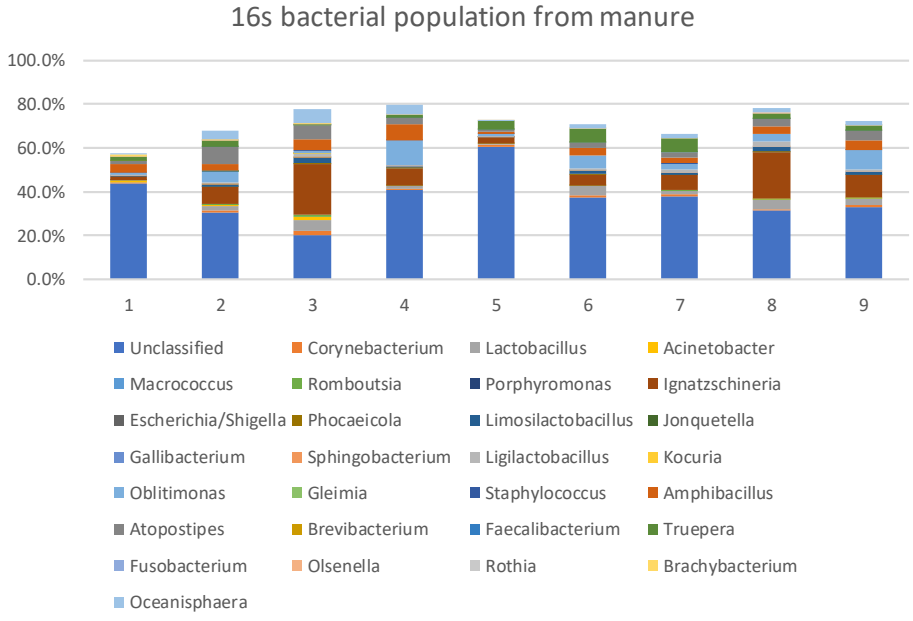
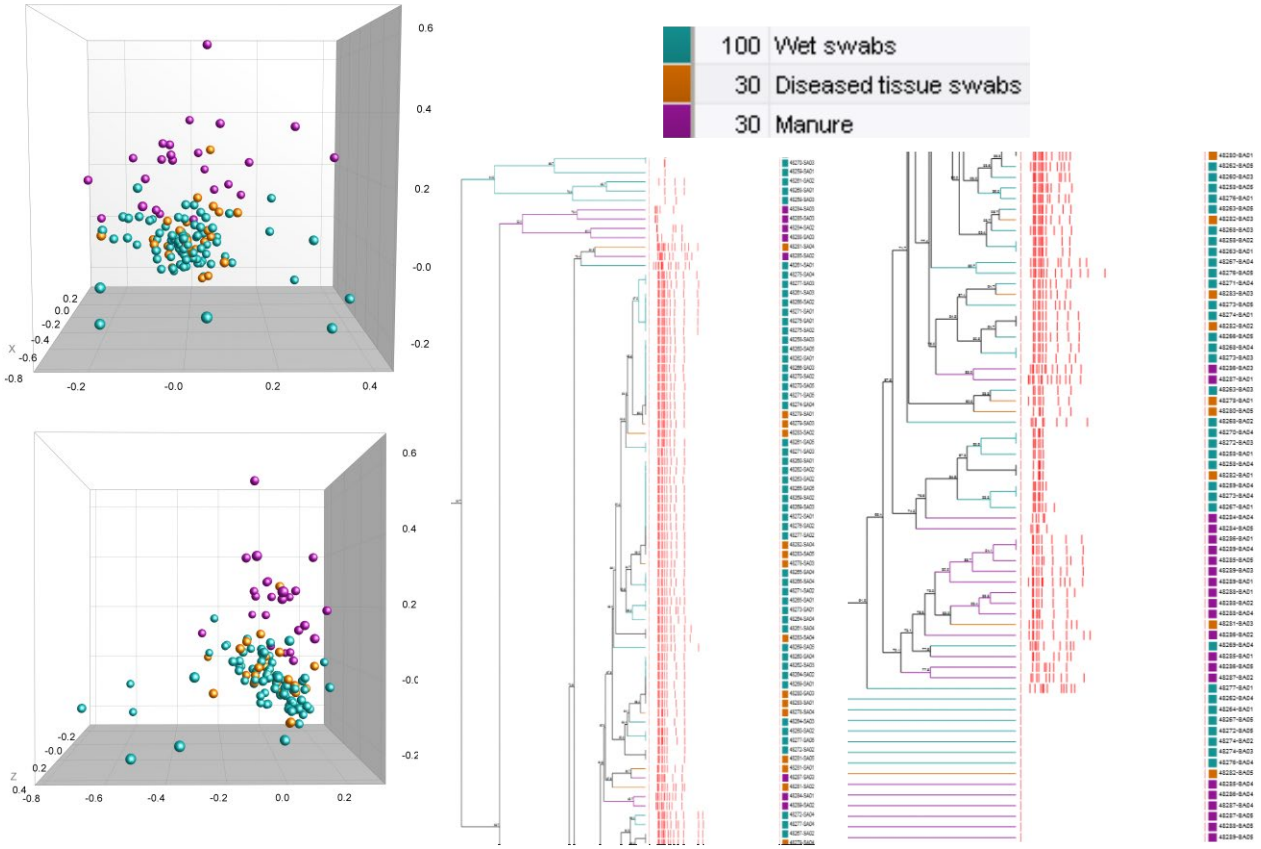


Figure 5. RAPD analysis of *M. caseolyticus* isolates. RAPD data is listed as both CPA of plot dendrogram data and the raw dendrogram data with relationships as connected bars. Colors used to indicate family of samples.



EARLY ONSET AND DURATION OF IMMUNITY OF A RECOMBINANT HVT-IBD VACCINE AGAINST VIRULENT, VARIANT, AND VERY VIRULENT INFECTIOUS BURSAL DISEASE CHALLENGES

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SUMMARY

Poulvac® Procerta™ HVT-IBD was developed as a bi-valent vaccine for protection against infectious bursal disease (IBD) and Marek's disease. Obtaining early IBD protection is important to reduce viral amplification and immunosuppression before flock immunity is achieved so flocks can reach their optimal potential. The vaccine showed 96-98% efficacy against classical virulent IBD and 95% protection based on bursa to body weight ratio (B:BW), which was 20-30% higher than market leaders. The vaccine showed 78% protection against variant AL-2 based on B:BW, and 90-93% protection against very virulent IBD. In broilers, with high maternal antibody (MDA), the vaccine showed 79.2-83.3% efficacy against vvIBD and 54% protection against variant AL-2 challenge once MDA waned. HVT-IBD also showed 100% protection against classical virulent and very virulent IBD following a Day 63 challenge. In conclusion, Zoetis's HVT-IBD vaccine showed strong and robust protection compared to market leaders against IBD challenges.

INTRODUCTION

Poulvac Procerta HVT-IBD was developed for protection against IBD and Marek's disease, two highly contagious diseases causing mortality, condemnations, and immunosuppression. Obtaining early IBD efficacy is vital to producers to reduce viral amplification and immunosuppression before flock immunity is achieved especially if maternal antibody is low due to a suboptimal breeder vaccination program or has waned due to high or early field challenge. However, even in flocks with high maternal antibody, strong flock immunity is needed once maternal antibody wanes and it is important that duration of immunity is maintained throughout the life of broilers. Classical virulent IBD protection is a key attribute of an effective vaccine but variant IBD protection is critical as ~50% of US broiler field isolates are AL-2 and vvIBD protection is important to prevent severe disease in high challenge regions such as California (1). This paper will present efficacy data for the Zoetis HVT-IBD vaccine for early classical, variant, and very virulent IBD efficacy, including a comparison to other HVT-IBD vaccines, and duration of immunity.

MATERIALS AND METHODS

Early classical virulent, variant AL-2, and very virulent IBD efficacy. SPF leghorn (CRL) eggs with no maternal antibodies, used to mimic a worst-case field scenario, were either *in ovo* (E18) vaccinated with 0.05 mL/bird or subcutaneous on Day 0 with 0.2 mL dose/bird. Vaccines were utilized targeting an equivalent dose within each comparator study. Birds were challenged on Day 14 via eye drop for the classical virulent (STC) and variant AL-2 challenges and on Day 12 via eye drop for the very virulent California IBD isolate. Birds challenged with classical virulent IBD were assessed based on IBD seroconversion, gross bursal lesions (edema and hemorrhage) four days following challenge, bursa to body weight ratios (B:BW) and histological lesions to assess lymphocyte depletion. Efficacy following variant AL-2 challenge was assessed for IBD seroconversion and B:BW ratios four days after challenge and very virulent IBD efficacy following challenge was based on histological lesion scores ten days following challenge. Challenged birds with a B:BW within 2 standard deviations of the mean B:BW of their respective non challenged controls 10 days post challenge were considered to be protected. IBD serological response was assessed via a ProFlok® IBD Plus Ab ELISA test utilizing a cutoff of 1,002 to determine positive birds. Histological lesions were assessed using the EP monograph 0587 bursal damage 0-5 point scale. Any birds that were scored with a histological lesion score less than 3 were considered protected. Studies with parameters assessed for significance was assessed at an α=0.05 level and studies had 80% power to show a 20% significant difference between treatment groups.

Very virulent IBD and variant IBD efficacy in high MDA broilers. Commercial broiler chickens

(Ross X Hubbard) eggs were either *in ovo* (E18) vaccinated with 0.05 mL/bird or subcutaneous on Day 0 with 0.2 mL dose/bird. Maternal antibody levels and serological response to the vaccines was assessed via an IDEXX IBD Ab test on Day 0 and Day 14-15. Vaccines were utilized targeting an equivalent dose within a comparator study. Birds were challenged via eye drop with either a very virulent California isolate challenge on 28 days of age and assessed via histological lesions 10 days post challenge or a variant AL-2 challenge on 22 days of age and assessed based on B:BW ratios 4 days post challenge as discussed above.

Duration of immunity. SPF leghorn (CRL) eggs were i*n ovo* (E18) vaccinated with 0.05 mL/bird or subcutaneous on Day 0 with 0.2 mL dose/bird. Serological response was assessed in the vvIBD study via a ProFlok IBD Plus Ab ELISA test utilizing a cutoff of 1,002 to determine positive birds on 14, 28, 42, 52, and 63 days of age. Birds were either challenged with either a classical virulent (STC) isolate and assessed for mortality and gross lesions 4 days post challenge or a very virulent California vvIBD challenge at 63 days of age and assessed for histological lesions 10 days post challenge.

RESULTS

Early virulent IBD efficacy. As expected, non-vaccinated challenged birds showed 100% of birds were susceptible to challenge based on gross lesions and B:BW ratio and 94.4% susceptible based on histological lesions. Following vaccination, the Zoetis HVT-IBD vaccine showed 96% (*in ovo*) to 98% (subcutaneous) efficacy which was numerically higher by 3-7% than Vaccine A and showed 23-25% higher protection compared to Vaccine B, which was significantly different. When assessing impact to bursa to body weight ratios, the Zoetis HVT-IBD vaccine showed 95% overall protection which was 25% and 35% numerically higher but not significantly different than Vaccine A and B, respectively. The Zoetis HT-IBD vaccine also showed 100% protection from histological lesions compared to 95% for Vaccine A and 75% for Vaccine B.

Early variant AL-2 efficacy. In SPF birds, the level of challenge take in non-vaccinated challenged birds was 100% for straight run birds and males and females. The Zoetis HVT-IBD showed the highest serological geometric mean titer (GMT) of 3,474 with 100% birds that had seroconverted by 14 days of age and showed 78% efficacy following challenge when males and females were combined. When males and females were assessed separately (data not shown), 62% of females were protected and 96% of males were protected. In comparison, Vaccine A showed 100% of birds that had seroconverted with a GMT of 3,130 but only 49% overall protection was obtained for males and females combined, which was significantly lower than the Zoetis HVT-IBD vaccine. When assessed separately in males and females, it provided 34% protection in females and 65% efficacy in males. These differences compared to the Zoetis vaccine were primarily due to decreased bursal weight in Vaccine A. Vaccine B showed a lower percentage of birds that seroconverted compared to the Zoetis vaccine, with 85% seroconversion on Day 14 with a GMT of 672. The percent of birds that were seroconverted was likely due to many of the birds GMT being closer to the assay cutoff. However, cell mediated immunity may have been present resulting in 71% protection when both sexes were combined, which was not significantly different than the Zoetis vaccine. Vaccine B showed a similar level of protection in males, with 94% protected but did have approximately 20% fewer females that were protected compared to the Zoetis vaccine. Like Vaccine A, the reason for lower protection in females, compared to the Zoetis vaccine, was due to lower bursal weights.

Broilers assessed for early variant AL-2 efficacy had a GMT of 19,792 at Day 0 but the GMT waned to 3,690 prior to the challenge at 22 days of age and 93% of non-vaccinated challenged birds (100% females and 86% males) were susceptible to challenge. The Zoetis HVT-IBD vaccine showed 54% protection following AL-2 challenge. The Zoetis HVT-IBD vaccine and Vaccine A showed a significant level of protection compared to the control. Vaccine A protected 53% of birds and Vaccine B protected 43% of birds but was not significantly different than the non-vaccinated control. In broilers challenged with vvIBD on Day 28, 95.8% birds showed clinical signs, over 50% lymphocyte depletion (histological lesion scores of ≥3), or mortality. The HVT-IBD vaccine showed 79.2% relative protection following subcutaneous vaccination and challenge and 83.3% protection following *in ovo* vaccination.

Early very virulent IBD efficacy in SPFs. Very virulent IBD results in severe clinical disease. As expected, all non-vaccinated challenged birds showed 100% of birds with severe clinical signs, histological lesions scores ≥ 3, and mortality in both of the studies utilized to assess the Zoetis HVT-IBD vaccine following *in ovo* and subcutaneous vaccination. When the Zoetis HVT-IBD vaccine was administered subcutaneous at day of hatch, it resulted in 90% efficacy and when it was administered *in ovo* at 18 days embryonation, the vaccine provided 93% efficacy. Thus, the vaccine shows strong protection against severe clinical disease.

Duration of immunity efficacy. Duration of immunity was assessed at 63 days of age for both

classical virulent and very virulent IBD. The Zoetis HVT-IBD vaccine protected 100% of birds from clinical bursal lesions following a virulent IBD challenge. In addition, when the vaccine was assessed for IBD seroconversion following *in ovo* vaccination, 74% birds seroconverted on Day 14 and 100% birds had seroconverted by 28 days of age through Day 63. On Day 63, birds had a GMT titer of 15,138 prior to challenge. The Zoetis HVT-IBD vaccine provided 100% protection following the Day 63 vvIBD challenge for both routes of administration.

CONCLUSIONS

IBDV remains one of the most challenging poultry health concerns[2]. Fast IBD protection is important to reduce viral amplification and immunosuppression before flock immunity is achieved to maintain body weight uniformity and prevent susceptibility to secondary infections so that flocks can reach their optimal potential. In the United States, the potential for IBDV infection in the first 3 weeks of age is high since many chickens are raised on built-up litter, and maternal antibodies passed down from hyperimmunized breeders begin to wane around 14 days of age.

While other challenges have become more common the past two decades, classic IBDV still can cause mortality rates of up to 5% (3) In comparison to Vaccine A, the Zoetis vaccine showed 3-7% numerically higher protection for gross lesions, 5% higher protection of histological lesions, and 20% higher protection based on bursa to body weight ratio following classical virulent IBD. In comparison to Vaccine B, the Zoetis HVT-IBD vaccine showed 25% higher efficacy based on classical virulent IBD histological lesions, 30% numerically higher protection based on bursa to body weight ratio, and 23-25% significantly higher efficacy based on protection from gross lesions.

The AL-2 variant has emerged as the most common IBDV challenge in U.S. broiler production, present in 52% of field samples recorded from 2014-2019 (1). Following variant AL-2 challenge, the Zoetis vaccine had 1% higher protection compared to Vaccine A and 11% higher compared to Vaccine B in broilers. Both the Zoetis vaccine and Vaccine A were the only two vaccines that were significantly different than the non-vaccinated control. In addition, the Zoetis vaccine showed 7% numerically higher protection compared to Vaccine B and 29% significantly higher protection based on bursa to body weight ratios following AL-2 challenge compared to Vaccine A.

While variant IBD challenges are the most common in the US, challenges from vvIBD are common globally, with the potential to cause mortality greater than 50% (3). Epidemiological research shows that 60% to 76% of IBDV isolates collected from four continents are vvIBD strains (2). The Zoetis HVT-IBD vaccine provided 90-93% efficacy as early as Day 12 which would have a large impact on reducing severe clinical disease. In addition to providing strong early protection, the Zoetis HVT-IBD also provided 79-83% efficacy in broilers with high maternal antibody once MDA began to wane at 28 days of age. In addition, the vaccine was able to maintain 100% protection from both virulent and very virulent IBD out to 63 days of age. In conclusion, the Zoetis HVT-IBD vaccine provided strong competitive protection both early and throughout the entire life cycle of broiler birds against all the key challenges (classical virulent, variant AL-2, and very virulent).

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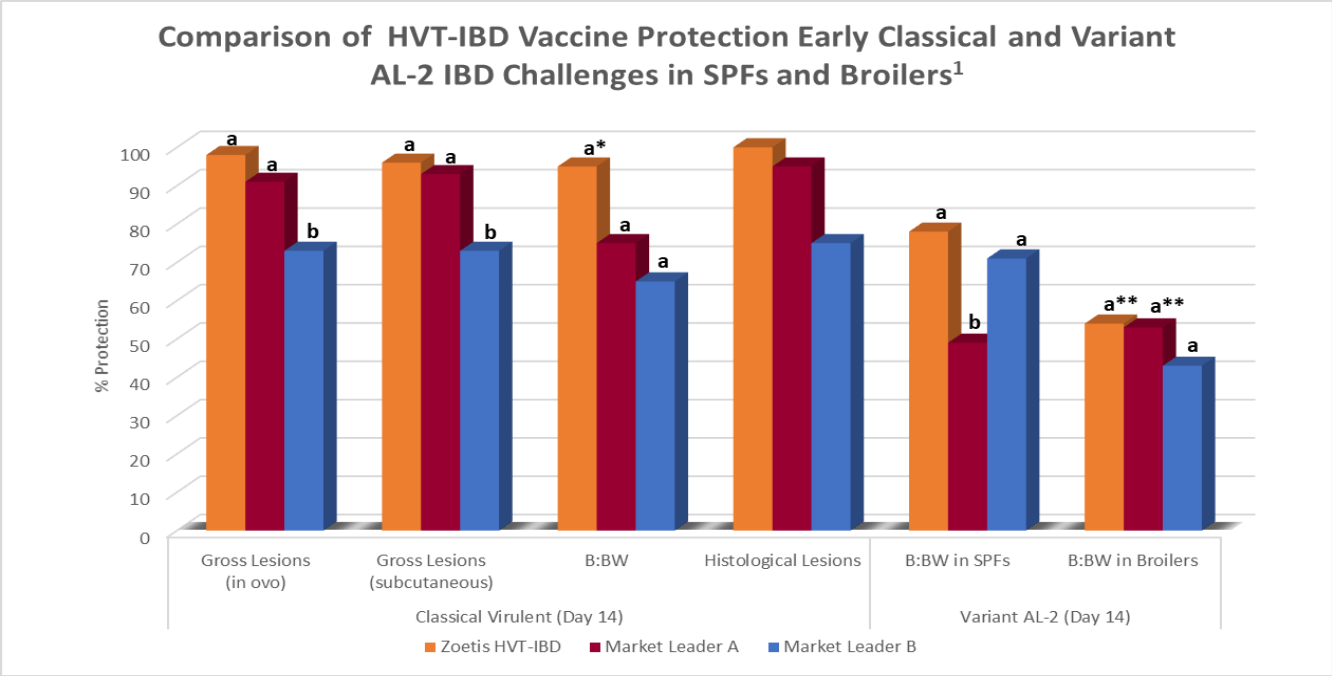
Figure 1.

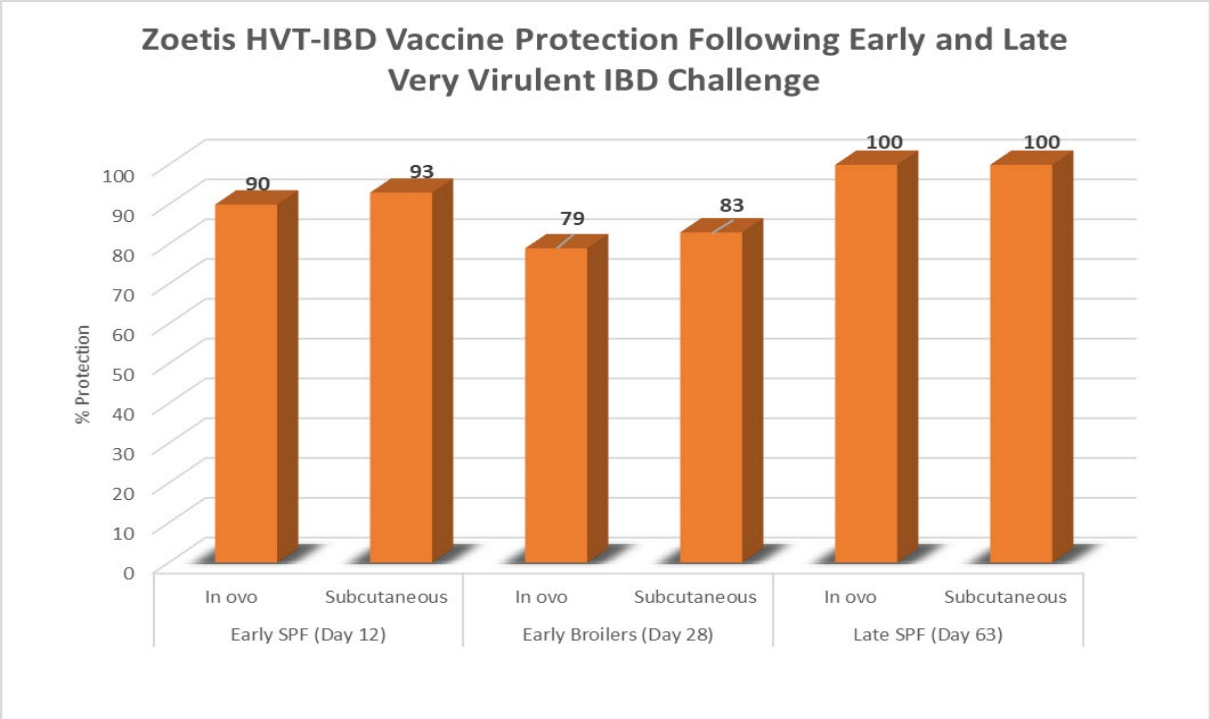


[1]Letters that are different in the same assessment are significantly different from each other at an α=0.05 level; studies had 80% power to show a 20% significant difference between treatment groups

* Zoetis HVT-IBD was not significantly different than either vaccine in males but was significantly different than Vaccine A for females

**Significantly different than the non-vaccinated challenge control

Figure 2.


Zoetis HVT-IBD Vaccine Protection Following Early and Late Very Virulent IBD Challenge
% Protection
100
90
80
70
60
50
40
30
20
10
0
90
93
79
83
100
100
In ovo
Subcutaneous
In ovo
Subcutaneous
In ovo
Subcutaneous
Early SPF (Day 12)
Early Broilers (Day 28)
Late SPF (Day 63)

DEVELOPMENT AND VALIDATION OF A NEW REAL-TIME PCR FOR SENSITIVE AND EFFICIENT DETECTION OF *ORNITHOBACTERIUM RHINOTRACHEALE* WITH COMPARISON TO THE CURRENTLY AVAILABLE REAL-TIME PCR ASSAY

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SUMMARY

Ornithobacterium rhinotracheale (ORT) infection leads to considerable economic losses in the turkey industry. Identification of the organism using routine bacteriologic examination is challenging due to the fastidious nature of the organism. The molecular diagnostics of ORT offers a good alternative. Currently, two PCR assays have been developed for the detection of ORT. One is conventional gel-based PCR (cPCR) and the other one is TaqMan real-time PCR (qPCR) assay. This qPCR assay was evaluated to be implemented as a diagnostic test in Iowa State University – Veterinary Diagnostic Lab. However, major flaws in primer and probe design were identified, which reflected negatively on the sensitivity and the efficiency of the assay. In this study, a more sensitive and efficient qPCR was developed and compared to the currently available assay. Compared to the currently available qPCR assay, the newly developed assay showed significantly better analytical sensitivity (approximately $1x10^3$ copies/mL) than the current assay ($3x10^6$ Copies/mL). Additionally, the new assay's efficiency (E=98.68%) was higher than the current assay's efficiency (E= 73.18%). The newly developed assay is an improvement on the currently available assay and will improve ORT detection from clinical samples.

INTRODUCTION

ORT is one of the most important bacterial respiratory pathogens facing the turkey industry (1, 2). In 2018, ORT was ranked to be third in the list of the top health issues in the US turkey industry (3). Depending on clinical signs and post mortem lesions are of little diagnostic value for ORT diagnosis since many other respiratory pathogens can produce similar clinical picture (4). Bacterial culture is the gold standard for laboratory diagnosis of bacterial diseases (5). However, the direct culture has its diagnostic limitations due to the fastidious nature of this microorganism. Therefore, detection of ORT often involves polymerase chain reaction (PCR), which is faster and more sensitive than bacterial culture (6-10). Currently, there is one available qPCR assay for the detection of ORT (11). This qPCR assay was evaluated to be implemented as a diagnostic test in Iowa State University – Veterinary Diagnostic Lab. However, major flaws in primer and probe design were identified, which reflected negatively on the assay's sensitivity and efficiency. In this study, we developed a more sensitive and efficient qPCR and compared the two assays' performance together.

MATERIALS AND METHODS

1. Target gene selection. The newly developed assay was designed to target the same conserved fragment of the 16S ribosomal ribonucleic acid (rRNA) that the previous assay is targeting. However, the primers and probe of the newly developed assay were placed in different locations on the segment to overcome the flaws of the previous assay primers and probe.

2. Primers and probes design and reaction conditions. Primers and TaqMan® probe were designed using Primer3Plus web interface (12). Subsequently, they were tested for their specificity through *in-silico* analysis using BLAST search tool (13) and then analyzed using the online IDT oligo Analyzer 3.1 tool (https://www.idtdna.com/calc/analyzer). All oligonucleotides (primers and probe) were synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA).

3. *Ornithobacterium rhinotracheale* isolates and clinical samples. Thirty-eight diverse ORT isolates along with twelve known ORT positive clinical samples, were included in the validation process. Thirteen samples from negative ORT flocks, using homogenate of tracheas and lungs from chickens and turkeys that are apparently normal, were also included.

4. Other bacteria and viruses. Twenty-three representative microorganisms (seventeen bacterial

pathogens and six viruses) that are likely to be found in samples submitted for ORT diagnosis were also examined as controls to check the qPCR assays' selectivity.

5. Evaluation of qPCR assays' performance. To validate the newly developed assay and compare its performance to the currently available assay, the following parameters were tested:

A. Selectivity. to measure the assays' reactivity against our target (ORT) and non-target microorganisms.

B. Evaluation of diagnostic specificity against clinical samples. Diagnostic specificity = true negatives/(true negatives + false positives) X 100.

C. Estimation of limit of detection (LOD) (analytical sensitivity) through the construction of ORT 16s rRNA positive control DNA. GBlock containing the forward, reverse primers and probe sequences were ordered from IDT (Integrated DNA Technologies, Coralville, IA, USA). The inserts were cloned into pCR® -Blunt II TOPO® (Invitrogen™) using manufacturer's recommendations. The plasmid was converted to a copy number after being quantified with the NanodropTM spectrophotometer. Subsequently, Ten-fold serial dilutions of the constructed positive control DNA containing ($1x10^{10}$ – 1x101) copies/mL were made to generate the standard curve, and 5µL was used as a template in each reaction. Amplification efficiency (E), coefficient of correlation (R^2), and dynamic range were estimated from the standard curve.

RESULTS

A new TaqMan probe-based qPCR assay for the detection of ORT was developed and validated. After that, a comparison between the currently available assay and the newly developed assay was performed.

Both assays could identify diverse isolates of ORT, indicating high inclusivity of the two assays. Simultaneously, the two assays showed 100% negative results against the panel of microorganisms that are likely to be found in samples submitted for ORT diagnosis. Moreover, the two assays showed diagnostic specificity equal to 100%.

The newly developed assay showed better efficiency (E= 98.68%) than the currently available assay (E=73.18%). The improved efficiency of the new assay subsequently improved the analytical sensitivity (LOD = 1000 copies/mL) than the current assay (106 copies/ml) (Table 1 and Figure 1). To explain the reason behind the current assay's reduced efficiency and to confirm if it is due to the primer-dimer formation, a melting curve analysis using DNA binding dye qPCR assay was performed. Two major peaks were detected, confirming the presence of primer-dimer formation.

CONCLUSION

The newly developed ORT qPCR assay is an improvement on the currently available assay and can serve as a reliable tool for the sensitive, specific and efficient diagnosis of ORT with the ability to test directly from clinical samples.

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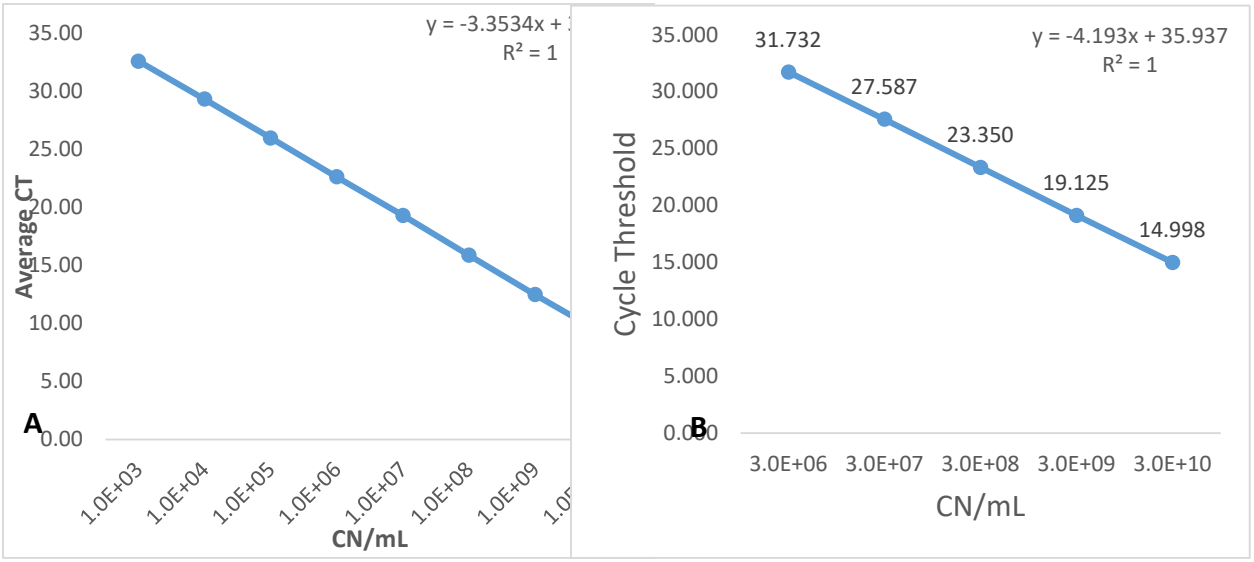
Table 1. Detection limits and standard curve results of the new and current qPCRs.

qPCR Assay	Amplicon Size	Limit of Detection	Linear Equation	R^2	Efficiency	Reference
New assay	131 bp	10^3copy/mL	y = -3.3534x + 36.013	$R^2 = 1$	E=98.68%	This study
Current assay	119 bp	10^6 copy/mL	y = -4.193x + 35.937	$R^2 = 1$	E= 73.18%	[11]

Figure 1. Standard curves of the two-qPCR assays included in this study



A: Standard curve of the new qPCR assay was generated by plotting average C_T values from three independent runs against $\log_{10}$ of 10 fold serial dilutions (10^{10} – 10^1) of plasmid DNA (copy number/mL). Reaction efficiency of 98.68% was estimated using the standard curve slope.

B: Standard curve of the current qPCR assay was generated by plotting average C_T values from three independent runs against log10 of 10 fold serial dilutions (10^{10} -10^1) of plasmid DNA (copy number/mL). Reaction efficiency of 73.18% was estimated using the standard curve slope.

DETECTION OF *HETERAKIS GALLINARUM* AND *HISTOMONAS MELEAGRIDIS* DNA IN DARKLING BEETLES AND LITTER AFTER DEPOPULATION OF CHICKEN HOUSES

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SUMMARY

Histomonas meleagridis is a flagellated ameoboid protozoan. The disease it causes is frequently fatal for turkeys. *Histomonas* dies within hours outside hosts. The primary reservoir facilitating survival of this pathogen in the environment and its spread is the cecal worm, *Heterakis gallinarum* (1). Cecal worms themselves are vector borne and also cause disease in poultry. They cause reduction in weight gain and feed efficiency in chickens (2). Several organisms are proposed as vectors of cecal worms and are thereby secondary order vectors of histomoniasis. Earthworms are paratenic hosts of cecal worms and Histomonas (3,4). Additionally, insects that feed on chicken excrement and litter can carry infected cecal worm eggs. In lab conditions, several insect species transmitted embryonated cecal worm eggs continuously for at least four days after an initial exposure (5,6).

Darkling beetles (*Alphitobius diaperinus*) are the most common insects in poultry houses (7,8). Cecal worm DNA was detected in darkling beetles by PCR, implicating them as vectors and/or reservoirs of cecal worms and thereby *Histomonas* (9). It is possible that litter beetles are a reservoir contributing to the persistence of both cecal worms and Histomonas through episodic emptying and cleaning of poultry houses.

The presented study characterizes the persistence of cecal worm DNA in darkling beetles in poultry houses for an extended period of time. Four houses at the Poultry Research Farm of Auburn University were sampled while in the process of decommission in the of summer 2020. Included were two broiler houses and two broiler breeder houses. Samples were taken 7 to 35 months after their most recent depopulation. Dead and live beetles were collected and tested individually by PCR for *H. gallinarum*. Pools of beetles and individual beetles were checked for *Heterakis* eggs by flotation. Litter samples consisting of at least 50 g were also collected and were mixed well before aliquots were taken for PCR and flotation. Samples were also investigated by PCR for *H. meleagridis*.

Beetles were identified as darkling beetles based on the morphology. Cecal worm DNA was consistently detected in both live and dead beetle corpses as well as in litter collected from the first house in which broiler breeders had been kept 16 months before the investigation began. In the second house, in which broilers had been kept nine months before, only one beetle was weakly positive, and in the third house in which broiler breeders had been kept 35 months before, several litter samples tested positive. No *H. gallinarum* DNA was detected in beetles and litter from the fourth house in which broilers had been kept seven months before sampling. In few beetles and litter, *H. meleagridis* DNA was detected. After flotation, one object resembling a nematode egg was found in a beetle pool collected in the first house. The object was perfectly ovule, had a chorion, and measured 25 µm x 40 µm, somewhat smaller than *H. gallinarum* eggs; the interior looked denser as in fresh eggs. Other objects, resembling eggs were also seen in the same sample. No similar objects were found in other beetle and litter samples.

The detection pattern was as expected, i.e. more positive samples came from broiler breeder houses than from broiler houses, and more recently used houses yielded more positive samples. This indicates that beetles did not spread significantly between houses. All samples types, live beetles, dead beetles and litter were positive to a similar degree, so there was no enrichment in beetles, but the potential of the beetles to act as mechanical vectors was clearly shown. The presence of *H. meleagridis* DNA was surprising, since there was never a recorded case of histomoniasis on the farm. In addition, the frequency of *H. gallinarum* eggs containing *H. meleagridis* is reported to be between one in 200 and one in 1,000 eggs only (10,11). However, the detection confirms widespread subclinical infection of chickens with *H. meleagridis* (12,13).

There was discrepancy between the relatively frequent *Heterakis*-positive samples as detected by PCR and the almost completely negative microscopy. One potential reason is that the eggs were detetoriating and not easily recognizable as *Heterakis* eggs. The

object we identified as a cecal worm egg was smaller and seemed to be denser, which might have been caused by dryness. Experiments in which eggs were stored in potassium dichromate or testing of infectivity of soil from contaminated outdoor plots, showed that eggs can remain viable for at least three years (14,15), but these results might not apply to dryer conditions in empty chicken houses.

In conclusion, cecal worm DNA and *H. meleagridis* DNA persists inside of or attached to beetles as well as in litter for months after depopulation of the house. The results indicate that darkling beetles act as a reservoir for a given house, but that there is limited exchange of beetles between houses, which will be important to implement suitable biosecurity measures. Further investigations will have to explore for how long the eggs remain infective under these conditions.

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

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CLOUDY WITH A CHANCE OF ENDOPHTHALMITIS

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SUMMARY

Seasonal occurrences of ocular lesions of unknown etiology had been occurring for three years during late spring and early summer. The lesions would occur approximately three weeks after meat-type pullets and cockerels were moved to the hen house. Lesions were more frequent in female birds than in male birds. Lesions were typically unilateral but could be observed bilaterally on occasion. Gross exam of the eye revealed corneal opacification progressing to severe inflammatory infiltrates observed in the anterior chamber of the eye progressing to caseous exudate. Culture of aspirates collected aseptically of the anterior chamber of the eye revealed *Cladosporium* and *Penicillium*. Eye swabs of the interior chamber of the eye yielded growth of *Streptococcus pluranimalium*, *Enterococcus cecorum*, *Penicillium*, and *Aspergillus fumigatus*. Fungal cultures of the litter revealed the isolation of similar fungal species with the addition of *Mucor* and *Rhizopus* spp. The full-length article will be submitted to Avian Diseases for publication consideration. All ocular samples were collected after humane euthanasia.

INTRODUCTION

Ocular lesions of commercially raised poultry are not a common occurrence. Ocular lesions of commercial poultry include infection with viral agents such as alphaherpesvirus (Marek's Disease) (1) bacterial agents: *Escherichia coli* (2), *Pseudomonas* (3) and *Salmonella* Arizonae (4) and mycoses associated with *Aspergillus* (5). Non-infectious causes include dust, dander and ammonia aerosol irritants, physical corneal lacerations, cataracts (6) and ocular tumors: rhabdomyosarcomas (7) from the iris's straited muscle, melanomas (1) and retinoblastomas (8,9) have also been reported. Gross pathology associated with alphaherpesvirus infection of the eye may cause "gray eye" due to the iris's loss of pigmentation, with irregularities to the margins of the pupil and other non-specific inflammatory changes (1). Panophthalmitis is seen when bacteria such as *E. coli* and *Pseudomonas* invade the ocular tissues (2, 3) *Salmonella* Arizonae infections in turkeys can cause hypopyon ophthalmitis (4). Mycotic ophthalmitis caused by *Aspergillus* can present either unilaterally (10, 11) or bilaterally (12). *Aspergillus* ophthalmitis can present in two forms: one infecting the external surfaces of the eye and tissues around the eye (13) or a second form involving the posterior of the eye including the vitreous humor and accompanied tissues (5). Clinical signs with the superficial form of mycotic ophthalmitis include keratitis, epiphora, periorbital swelling, swollen eyelids adhering together and turbid discharge (13). Mycotic ophthalmitis involving the posterior eye is often associated with a systemic respiratory infection precipitating a hematogenous spread of *Aspergillus* to other tissues including the deeper sections of the eye (5).

A meat-type breeder operation had been experiencing seasonal occurrences of ocular lesions of unknown etiology in the late spring and summer. The ocular lesions were progressive in nature appearing approximately three weeks after moving from the pullet farm to the layer house. This has been a recurring issue; therefore, more detailed diagnostics were performed to elucidate the possible etiologic agent(s).

Birds with a cloudy eye began to be observed by service persons during routine walk through and handling of flocks. The cloudy appearance, to the eye, would progress to suppurative exudate, caseous exudate with blindness in the affected eye and possible rupture of the eye. Hens were more often affected than roosters. The lesions were mostly unilateral. The ocular opacities were not observed in the pullet house. The cloudy appearance to the anterior chamber of the eye began to appear approximately three to four weeks post-move, housing in the lay/breeder houses. The lesions, when unilateral, did not affect the fleshing, weight or productivity of infected hens. Subjectively, the lesions did contribute to a minor increase in mortality through approximately 35-weeks-of-age. Daily and weekly mortality was not segregated nor recorded based on pathologic lesions or causes of mortality. Poultry house bedding is sourced locally from mostly hardwood, freshly ground trees. Shavings are often damp when placed into the houses. Pullet houses' bedding is changed once a year or every two grow-outs and breeder house shavings in the scratch area is changed with every flock. Different breeds did exist on the same breeder farm. Sample size was too small to make any scientific conclusions.

MATERIALS AND METHODS

Production and mortality records were reviewed. Gross examination consisted of walking the barns, observing litter conditions, birds and bird behavior. Five clinically affected birds with ocular lesions were humanely euthanized from one house on each breeder farm. Three samples from the eyes were taken: ocular fluid (anterior chamber and vitreous humor), ocular swab of posterior of the eye and eye tissue for histopathology.

Ocular fluid was obtained aseptically using a sterile needle and syringe inserted into the anterior chamber and aspirated. Without removing the needle, the needle was extended through the lens and inserted into the posterior chamber where an aspirate of the vitreous humor was obtained. This fluid was aseptically transferred to a sterile red top, no additive, blood collection tube and chilled. Samples were taken individually but pooled at the diagnostic lab due to the limited amount of fluid. Aerobic bacterial culture and fungal culture were performed on the pooled ocular fluid.

Swabs of the posterior chamber of the eye were collected using a small incision in the cornea and a sterile swab was inserted into the posterior chamber of the eye globe. The swabs of posterior chamber fluid were individually placed in brain heart infusion and chilled. Brain heart infusion media was pooled for aerobic culture and fungal culture at the diagnostic lab.

The final sample taken was the entire ocular globe. The contralateral eye from the eye in which ocular fluid and swabs were taken was enucleated and placed in 10% neutral buffered formalin. A small incision near the optic nerve attachment was made to allow rapid fixation of the interior structures. Standard histologic tissue preparation was performed. Stains used were Hemotoxin and Eosin (H&E) and Periodic acid-Schiff (PAS).

Litter samples were collected in five evenly distributed locations within each house from which clinically affected birds were sampled. Litter samples were cultured for mycotic species using Sabouraud dextrose agar. Quantification of mycotic species was not performed.

RESULTS

Two geographically separate farms were visited. Farm A at 29 weeks of age and Farm B at 31 weeks of age. Farm A, 29 weeks of age, had approximately 20% of the hens affected with varying degrees of cloudy corneas, hypopyon, endophthalmitis with opaque debris in the anterior chamber of the eye to caseous exudate with panophthalmitis (Figure 1). The ocular lesion was most often unilateral with rare occurrences of bilateral endophthalmitis. A menace response was performed on several birds with involved endophthalmitis. The menace response was negative, no menace. In advanced lesions with panophthalmitis, the menace response was also negative. On rare occasion, unilateral endophthalmitis and panophthalmitis could be observed in roosters. Farm B, 31 weeks of age, had a lower relative incidence of near 10%. Ocular lesions, cloudy cornea, hypopyon, endophthalmitis, and panophthalmitis were as described at Farm A. At both farms, physical exam of birds revealed well fleshed, well hydrated, well feathered, feed in the ingluvies, and a hen in production. Necropsy of the internal celomic cavity did not yield any gross pathology including the lungs.

Examination of the house and equipment did not reveal any possible injury points, rough areas of the hen feeders that may cause a corneal laceration with secondary infection. No new equipment had been installed and this was not the first flock raised on Farm A or Farm B.

Farm A. Bacterial aerobic culture of eye fluid resulted in no significant growth. Aerobic bacterial culture of the eye swabs cultured heavy growth of *Streptococcus pluranimalium* and *Enterococcus cecorum*. Mycotic culture of eye fluid was positive for few *Penicillium* spp. colonies. Eye swabs cultured a single fungal colony of *Aspergillus fumigatus*. Heavy growth of *Penicillium*, *Mucor*, and *Rhizopus* was present from the litter sample from Farm A.

Farm B. Bacterial aerobic culture of eye fluid and eye swabs resulted in no significant growth. Mycotic culture of eye fluid was positive with few colonies of *Cladosporium* spp. Eye swabs cultured a few fungal colonies of *Penicillium*. Heavy growth of *Penicillium* and *Mucor* were cultured from the litter samples.

Histopathology of the eyes revealed similar lesions for all eight eyes, four from each farm. Microscopic exam revealed scattered to multiple aggregates of fungal hyphae within and attached to the cornea. The mycelium was 4 to 6 microns wide, septate, fairly uniform diameter with branching but had no conidial heads (Figure 2). There were multiple aggregates of heterophils within the cornea, iris, and ciliary bodies. Morphologic Diagnosis: Severe, multifocal, mycotic keratitis with heterophilic panophthalmitis.

DISCUSSION

The changes in the eyes are compatible with a fungal infection. Cultures of the eyes revealed *Cladosporium* spp. and *Penicillium* spp. from Flock B, and *Penicillium* spp. and *Aspergillus fumigatus* from

Flock A. Fungal cultures of the litter had *Penicillium* spp. and *Mucor* spp. from Flock B, and *Penicillium* spp., *Mucor* spp. and *Rhizopus* spp. from Flock A. The fungal agents in the affected eyes are very similar in morphology and without the conidial heads difficult to differentiate from *Penicillium* spp. and *Aspergillus* spp. *Penicillium* spp. is detected in both flocks' litter samples and within the eyes of both flocks' birds.

The clinical presentation of breeder birds, mostly hens, with unilateral opaque corneas, hypopyon, endophthalmitis and panophthalmitis had been occurring seasonally over the last three late springs early summer. Upon first presentation it was believed that ocular infection was occurring during oculonasal vaccination at 11-weeks of age in the pullet house from minor corneal abrasions. This was ruled out when pullets were examined, and no pullets or cockerels presented with the lesions of interest. An alternative hypothesis also included corneal abrasion and subsequent opportunistic pathogen invasion. The lesion tends to first appear about three weeks after the pullets and cockerels are moved to the hen/lay house. Could there be something in the moving process be leading to corneal abrasions in a subset of the population? This hypothesis was ruled out as birds are moved with the same equipment and in the same manner all year long. Additionally, these birds are moved using standard industry practices and procedures which have not changed in years.

This breeder operation is in an area where the hens' houses have supplemental heat. The litter is mainly of hardwood material, is often ground on demand thus shavings are often damp upon receipt and placement in the houses. However, the same supplier and same litter material is used for pullets and the lesions are not appreciated in the pullet houses. One management practice is not needed during the late spring and summer. This management practice is the use of supplemental heat during the warmer months of year. Conversely, during the late spring and early summer, heat is trying to be removed from the houses.

How does this play into mycotic endophthalmitis and panophthalmitis? Pullet houses receive new litter every other grow-out/flock. All pullet houses begin being pre-heated to 90-degrees Fahrenheit 48-hours prior to pullet and cockerel chick placement. Additionally, during this time, the houses are minimum ventilated removing combustion gases and ammonia in the case of used litter being used. Pre-heating and ventilation allow for drying of the litter minimizing available water to support microbial and mycotic growth prior to bird arrival.

In the hen/lay house, houses are heated to desired set points for 22-week old pullets and cockerels during the colder months of the year. This too allows for drying of the litter, in the scratch area, prior to bird placement. During the late spring and summer, ambient environment temperatures are high enough that the litter does receive supplemental heat to aid in hastening the drying process.

The authors' hypothesis of mycotic endophthalmitis and panophthalmitis being a seasonal incidence lies in the amount of available water in the litter at placement coupled with the hardwood sources being higher risk litter materials for mycotic growth. Furthermore, corneal ulceration is not a likely route for environmental and opportunistic bacterial or fungi to enter the anterior and posterior chambers of the eye. The most likely route of ocular infection is through aerosol inhalation of mycotic spores from the litter, infection of the lung parenchyma with hematogenous spread to the ocular chambers and tissues. This route of infection is supported by other authors especially with *Aspergillosis*[5]. If these lesions present again, collection and histopathologic examination of the lungs will be included in the diagnostic work-up. *Streptococcus pluranimalium* and *Entercoccus cecorum* are likely contaminants during the collection process as these are common bacteria in poultry rearing areas.

Aspergillus, *Penicillium*, *Cladosporium*, *Mucor*, and *Rhizopus* are all common fungi of wet environments, soils, hardwood and certain feedstuffs. *Cladosporium* are ubiquitous and are found on plant debris, soil, and other various organic materials (14). *Cladosporium* spore detachment has been reported to increase when wind speeds reach or exceed one meter per second which is greatly exceeded during tunnel ventilation (15). *Aspergillus* is a well-known mycotic species in poultry. *Aspergillus* is commonly found in decaying vegetation, soils, and seeds and grains[16]. *Penicillium* too, is found in soils, feedstuffs, grains and has a wide distribution (17). The Mucorale Zygomycetes *Mucor* and *Rhizopus* are cosmopolitan and found in soil and plant debris (18). Due to the wide distribution and occurrence of these fungi in soils and on plant materials it was not

Therefore, the ocular infections are a likely sequalae of the environment, litter source and water content of the litter. Resolution and mitigation could include using a different litter source and relying on softwoods such as pine shavings, purchase kiln-dried hardwood savings, institute an in-house drying process when damp shavings are received or treating the litter. Being as though these are food producing animals, treating the litter is not a likely option and is discouraged.

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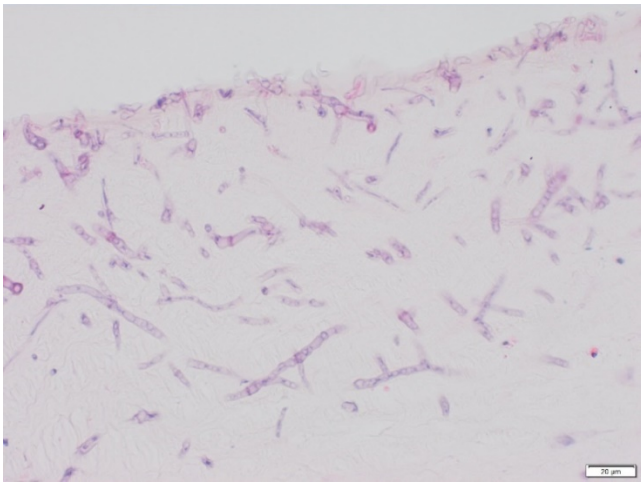
Figure 1.

Figure 2.



A CASE OF HEMORRHAGIC ENTERITIS IN GROWING TURKEYS

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SUMMARY

Two turkey houses on the same premise reported sudden onset of increasing mortality affecting 10 to 13-week-old Nicholas turkey hens. The birds came from a premise that held 90,000 birds and each house held 10,100 birds. Clinical signs included feather and litter eating, and enteritis, however, the birds appeared otherwise normal. Treatment administered included copper sulfate in the drinking water. On post mortem examination, moderately to markedly enlarged spleens with multifocal hemorrhage and necrosis, myocardial hemorrhage and necrosis, edematous lungs and diffusely congested duodenum, Peyer's patches and cecal tonsils were seen in the majority of birds. Bacterial cultures were negative from the spleen, liver, heart, lung and airsac. On histopathology, extensive lymphocyte depletion, necrosis and hemorrhage, with large, lightly basophilic intranuclear inclusions were present in the spleen. PCR tests for hemorrhagic enteritis virus was positive. The birds were negative for avian influenza, reovirus, *Mycoplasma gallisepticum*, and *Mycoplasma synoviae* by PCR method. Hemorrhagic enteritis virus is an acute disease of growing turkeys, which results in enteritis caused by *Siadenovirus*. Protection is achieved through vaccination. On recheck, three weeks later the flock was in excellent condition and mortality was back to normal levels.

INTRODUCTION

Hemorrhagic enteritis (HE) is an economically important disease of turkeys caused by virulent strains turkey adenovirus A (Turkey adenovirus 3, TAdV-3) of the *Siadenovirus* genus. Infection produces an acute viral disease in turkeys greater than 4 weeks of age, with clinical signs include diarrhea, which may be bloody, depression and death (1). The clinical course of the disease is 7-10 days, however, the immunosuppressive effects of the virus, predisposes infected birds to secondary bacterial and viral infections resulting in protracted periods of losses (1, 2). Poor growth rates, and impaired feed conversion efficiency lead to economic losses. Turkeys, 6-11 weeks of age are most susceptible, and birds younger than four-weeks-old are considered refractory to infection due to protection form maternal antibodies (1, 3). Transmission is horizontal via the oral or cloacal route, and vertical transmission is not a feature of this viral infection (4). The disease progresses rapidly in a flock with bloody droppings, depression and death (1, 3, 4). Gross lesions include enlarged, friable, marbled spleens, congested lungs, and reddened intestinal mucosa. On microscopic examination of the spleen, lymphoid necrosis, with basophilic intranuclear inclusions are characteristic findings (1). Additional diagnostic methods include immunofluorescence, immunohistochemistry, real time PCR, ELISA, and *in-situ* hybridization. Control is achieved through biosecurity, hygienic management practices and vaccination.

CASE REPORT

Three live and four dead, ten to thirteen-week-old, Nicholas turkey hens, were submitted to the California Animal Health and Food Safety Laboratory System, Turlock branch for necropsy. The birds came from separate houses on the same ranch, which held ninety thousand birds. Each house held approximately 10,000 birds that were outdoor reared. The history included mortality of 185 birds per day and each house had approximately 30 birds that appeared sick. Over the course of three days, the mortality increased from 33, to 64, 129, and 185 on the day of submission. Clinical signs included litter eating, and diarrhea, however, the birds were otherwise bright and alert. Two days prior to presentation, treatment of copper sulfate was added to the drinking water. The birds received no prior vaccinations.

On post mortem examination, the birds were in excellent body condition, and mild dehydration was evident. The most prominent lesion was markedly enlarged spleens, with coalescing dark red foci reminiscent of marbling (Figure 1). All birds had prominent, kidneys and roughly half of the birds had coalescing white foci on the heart, most pronounced on the right ventricular myocardium. The lungs were diffusely wet and oozed serosanguineous fluid. The duodenal mucosa of all birds was diffusely hyperemic and there was segmental reddening of the jejunal mucosa. Peyer's patches and cecal tonsils were diffusely dark red. Microscopically, in the spleen,

coalescing necrosis of the white pulp, with edema, hemorrhage and severe lymphoid depletion was evident (Figure 2). Numerous lymphocytes contained large, basophilic intranuclear inclusions, which distorted the nuclear chromatin to the periphery (black arrows). The heart had multifocal myocyte necrosis with diffuse lymphoplasmacytic infiltrates and lymphoid nodule formation. Multifocal centrilobular hepatic necrosis was present in the liver. Mild, multifocal hemorrhage was seen in the lamina propria of the small intestine. Aerobic cultures of the liver, heart, lung, spleen and airsac revealed no growth after 48 hours. Polymerase chain reaction (PCR) for avian paramyxovirus-1, avian influenza and reovirus were negative, however, the Turkey hemorrhagic enteritis virus PCR was positive from the splenic tissue (CT value of 17.75). The birds were serologically negative for avian influenza, *Mycoplasma gallisepticum*, *Mycoplasma melegridis*, *Mycoplasma synoviae,* and Newcastle disease virus. Virus isolation for reovirus from the heart tissue was negative.

DISCUSSION

Hemorrhagic enteritis was diagnosed in the flock based on the history, gross and microscopic findings and the positive PCR results. Based on the acute onset of high mortality, PCR testing for avian influenza and virulent Newcastle disease was placed on priority status to rule out these notifiable diseases. Some other differentials for this condition include bacterial septicemia such as from *E.coli*, *Pasteurella multocida*, and *Erysipelothrix rhusiopathiae*, which produce enlarged mottled spleens and high mortality (1). Lymphoid neoplastic diseases such as reticuloendotheliosis and lymphoproliferative disease should also be considered (1). The immunosuppressive effects of TAdV-3 predisposes affected birds to secondary infection, however, on microbial cultures no bacteria were isolated from the spleen, liver, lung or heart. One interesting finding was myocardial necrosis affecting few birds, with centrilobular hepatic necrosis suggesting heart failure. The underlying cause of the heart lesions was not determined, and reovirus PCR and virus isolation from the hearts was negative. These birds were most likely susceptible to infection to TAdV-3 because they were reared outdoors and were not vaccinated. On re-check three weeks after submission, the birds appeared healthy and mortality decreased to normal levels.

(The full-length case report will be published in *Avian Diseases*.)

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Figure 1

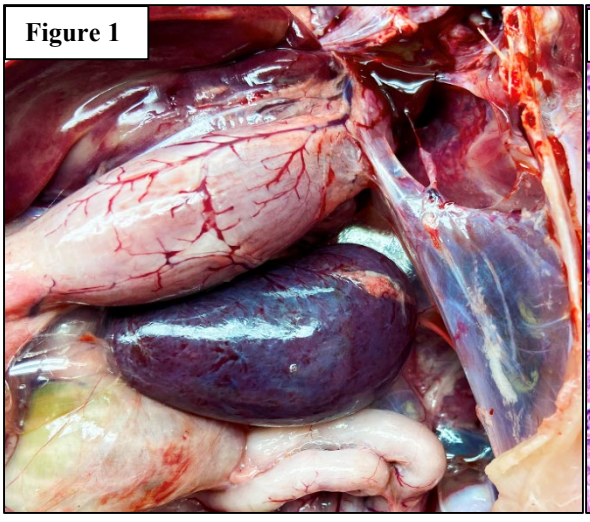
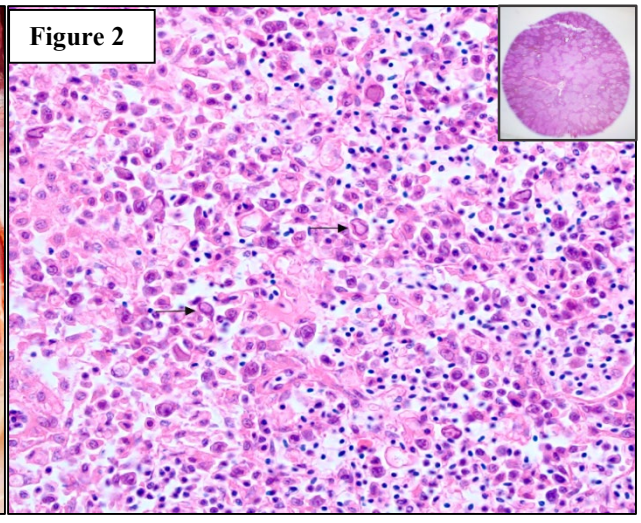


Figure 2



THE USE OF QUADRIVALENT CORYZA VACCINE TO ACHIEVE BROAD PROTECTION AGAINST FIELD ISOLATES IN INDONESIA

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SUMMARY

Infectious coryza is a respiratory disease caused by *Avibacterium paragallinarum*. During 2009-2019, we isolated a total of thirty-nine positive samples from layer chickens in Indonesia, of which 20.5% identified as serogroup A and 79.5% as serogroup C based on hemagglutination-inhibition (HI) test. Sequencing analysis revealed that the majority of recent field isolates were serovar C-4 in Kume classification. Most coryza vaccines combining two to three serogroups (A, C-2, with/without B) from universal vaccine strains cannot produce adequate cross-protective antibodies against the current C-4 field isolates. The addition of a C-4 isolate in the quadrivalent vaccine produces more HI antibodies and protection against challenges. Our study in commercial layer chickens shows high HI titer for A, B, C-2, and C-4 after two-doses of vaccination. The high antibody titer lasted for more than seven weeks after the second vaccination in commercial layer chickens. No side effect was observed during fifteen weeks post-vaccination.

INTRODUCTION

Infectious coryza is an upper respiratory disease caused by Avibacterium paragallinarum affecting poultry, with high morbidity (up to 20%) but low mortality (1,2) (1, 2, 3). Clinically infected chickens show unilateral or bilateral eye discharge, facial cellulitis, diarrhea and chronic sinusitis. Acute and chronic infection results in drop of egg production and performance, more culled chickens, and increased cost for medicine (2).

Page divided Avibacterium paragallinarum (Ap) into three serogroups: Ap A, Ap B, and Ap C. Kume then developed nine serotypes from the Page's serogroup classification using agglutination test. There is no adequate cross-protection among serogroups; protection among serotypes is low as well (A1, A2, A3, A4 or C1, C2, C3, C4) (3). Vaccination failure can be caused by low cross protection from vaccine strain against field strain. Our previous study in Indonesia showed that serogroup C is predominant; therefore, better protection against C4 is required.

MATERIALS AND METHODS

Nasal discharge and nasal swab were cultured in selective Chocolate Agar medium and incubated to select the field isolates. These isolates were serotyped using Page scheme and HI test. The hemagglutinin serovar of each isolate was assigned according to the highest HI titre. DNA sequencing was conducted from the gene expressing HMTp210.

Cross protectivity was determined by vaccinating chickens using monovalent vaccines containing serotype C (two doses: at age five weeks and eight weeks) via intramuscular injection. Vaccinated chickens were challenged with homologous and heterologous serotype C field isolates at two weeks after the second vaccination. Challenge was conducted with 0.2 mL of field isolates, dose $\geq 1 \times 10_8$ CFU via nasal drop. Safety and potency of quadrivalent vaccine (MCQ - combination of serotype A1, B, C2 and C4) were evaluated using the same method as above. Blood samples from chickens are drawn at 9 and 11 weeks after second vaccination and antibody titer was measured by HI titration. Clinical signs (runny nose, swollen face, watery eyes) were observed every day for 10 days post-challenge.

RESULTS

During 2009-2019, a total of thirty-nine positive samples from layer chicken in Indonesia were isolated, of which 20.5% was identified as serogroup A and 79.5% as serogroup C based on the hemagglutination-inhibition (HI) test. DNA sequencing analysis revealed that the majority of recent field isolates were serovar C4 in Kume classification (Table 1 and Figure 1).

Monovalent vaccines containing either serotype C2 (strain Modesto) or C4 (field isolate) gave the best protection against challenges using various field isolates. They also gave relatively high HI titer but HI titer from chickens vaccinated with the quadrivalent vaccine (MCQ) was above standard for serotype A1, B, C2 and C4. The titer was still at increasing trend at 6 weeks after vaccination. The percent immunity was above standard at three weeks post-vaccination. Challenge test result shows that MCQ is protective against serotype A, B, C2 and C4. The GMT value of HI titer against C4 is higher from the quadrivalent

vaccine MCQ than the trivalent vaccine. The high antibody titer lasted for more than seven weeks after the second vaccination in commercial layer chickens and remained above standard until 10 weeks. No side effect was observed during the fifteen weeks post-vaccination.

CONCLUSION

Most coryza vaccines containing a combination of two to three serogroups (A, C-2, with/without B) from universal vaccine strains cannot produce adequate cross-protective antibodies against the currently circulating C-4 field isolates. The addition of a C-4 isolate in the quadrivalent vaccine produces higher antibodies and giving better protection. Our study in commercial layer chickens shows high HI titer and full protection against serotype A, B, C-2, and C-4 after two doses of vaccination.

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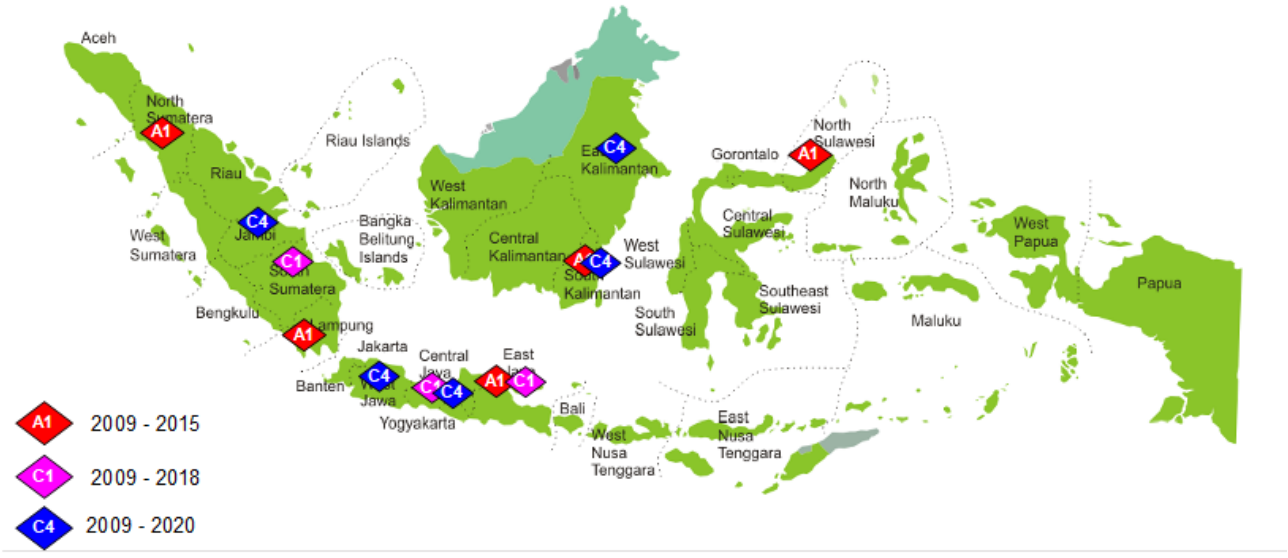
Table 1. Serotypes of field isolates obtained.

Serotype	Number of isolates	Year
A1	8	2009 - 2015
C1	2	2009 - 2018
C4	29	2009 - 2020

Figure 1. Map of Indonesia showing isolate origin and their serovars.



REDUCTION IN TITER OF INFECTIOUS BRONCHITIS VIRUS VACCINE WHEN COMBINED WITH COCCIDIOSIS VACCINES

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SUMMARY

Infectious bronchitis (IB) and coccidiosis are two of the most challenging diseases in poultry production and are commonly controlled by vaccination. The live vaccines are both commonly applied in the hatchery using spray cabinets, where coccidia vaccines are applied in a 21 mL volume, and infectious bronchitis virus (IBV) vaccines are applied in 7 or 14 mL volumes. When both vaccines are applied separately, the volume sprayed onto the chicks can be significant and may cause over-wetting, which could potentially chill chicks. To avoid this, combining both vaccines into one spray application has been proposed. In this study, we evaluated the effect of the composition of coccidiosis vaccines (with or without a potassium dichromate preservative) on the titer of live attenuated IBV vaccines over time, as if they were being mixed for a combined application in a hatchery. The results of this study show that there was a negligible loss in titer for combinations of an IBV vaccine with coccidiosis vaccines without potassium dichromate. A reduction in mean IBV vaccine titer of 0.5 log EID_{50} was observed when combining with coccidiosis vaccines that do contain potassium dichromate, however, a statistically significant reduction in IBV titer at any individual time point was not observed for any of the mixtures. This indicates that it is reasonably safe to combine coccidiosis vaccines that do not contain potassium dichromate with IBV vaccines for streamlined mass application, but considerations should be made prior to combining coccidiosis vaccines with potassium dichromate with IBV vaccines.

INTRODUCTION

IB and coccidiosis are two of the most economically significant diseases in poultry production. IB is caused by IBV and is characterized by snicking, rales, conjunctivitis, tracheitis, decreased egg production, and misshapen/wrinkly eggs (1). Cases of IB are often further complicated by bacterial infections that lead to airsacculitis, which results in increased condemnation of poultry meat and slower line speeds at processing (2). It has been suggested that an IB outbreak can cost upwards of $450,000 per week for an operation producing 1 million, seven-pound broilers per week when all factors are considered (3).

Coccidiosis is an enteric disease of commercial poultry caused by protozoal parasites of the *Eimeria* spp., with decreased performance and worsened feed conversion ratios being the most common outcomes of infection (4). Some species that infect commercial poultry may also cause mortality in severe cases. A primary sequela to coccidiosis is the development of necrotic enteritis, caused by *Clostridum perfringens* (5). This condition can be a significant cause of mortality, especially in operations that are antibiotic free and have limited treatments for this condition.

The main method of control of IB in broilers is live attenuated IBV vaccines delivered in the hatchery on day of hatch, while control of coccidiosis has been primarily with anticoccidial drugs and ionophores. As drug resistance has increased and antibiotic free/organic programs have become more popular, vaccination in the hatchery has become increasingly more common (6). IBV vaccines are often combined with other vaccines (Newcastle disease virus, etc.) at application, but have not been combined with coccidia vaccines due to the routine inclusion of a dichromate chemical with antibacterial and antiviral properties (7). Using both vaccines separately however may lead to over-wetting and chick chilling since the total applied volume would be large (at least 28 mL). Several companies are now also applying a *Salmonella* vaccine in spray cabinets, which can further increase the total volume.

Recently, several coccidia vaccine manufacturers have removed the dichromate chemical from their final coccidia vaccine product, making it possible to potentially combine coccidia and IBV vaccines to reduce the total vaccine application volume and streamline the vaccination process. The effect of these new coccidia vaccine formulations on IBV vaccine titers has not been evaluated however, so combining vaccine is not currently the standard practice. The aim of this study was to test the effect of different coccidiosis vaccines on the titer of IBV vaccines over time when in combination, to determine

the feasibility of a combined application. Additionally, since mixing concentrated coccidia vaccines containing dichromate with IBV vaccines may negatively impact the viability of the IBV vaccine, we examined the potential effect of dichromate on the titer of IBV vaccine when mixing concentrated forms of the two vaccines prior to diluting the vaccine mixtures to full application volumes.

MATERIALS AND METHODS

Vaccines. In this experiment Mildvac Ma5 (Merck Animal Health) IBV vaccine was used in combination with the following coccidiosis vaccines: Hatchpak Cocci III (Merial/BI), Immucox I (Ceva), Coccivac B-52 (Merck Animal Health), Advent (Huvepharma), and InOvoCox (Huvepharma). Immucox I and Hatchpak Cocci III are the only coccidiosis vaccines that were tested in this trial that use potassium dichromate as a stabilizer.

Vaccine mixture preparation. Experiment 1. In this experiment two vaccines were titrated. The first was the lyophilized Mildvac Ma5 IBV vaccine alone. It was reconstituted with 10 mL cold, sterile deionized water, and then diluted to 100 doses per 21 mL (to mimic the combined application volume). The second was a mixture of Mildvac Ma5 IBV vaccine and one coccidiosis vaccine. In this experiment, HatchPak Cocci III and Immucox were each tested individually with a separate dilution of Ma5. Coccivac B52, Advent, and InOvoCox were all tested at the same time against the same dilution of Ma5. For the IBV and coccidiosis vaccine mixture, both vaccines were mixed into cold, sterile deionized water at the same time to give a final concentration of 100 doses per 21 mL of each vaccine. Once prepared, the vaccine mixtures were stored in the refrigerator for the duration of the experiment.

Samples of each experimental group (IBV vaccine alone and IBV vaccine mixed with coccidia vaccine) were taken at mixing, and then every 30 minutes for 4 hours for titration. For each time point for each experimental group, the vaccine was hand mixed thoroughly in a 4 L Erlenmeyer flask and 100 mL was removed and filtered through a 0.22 um Millipore Express Plus (EMD Millipore, Billerica, MA) vacuum filter system. The filtration was done to remove the coccidia oocysts and leave only IBV so that the oocyst would not affect the IBV titration in embryonated eggs. Following filtration, 1 mL was removed for titration.

Experiment 2. To investigate the potential effect of dichromate when mixing concentrated forms of the two vaccines prior to diluting the vaccine mixtures to full application volumes, Hatchpak III and Immucox I, the two coccidiosis vaccines that contain potassium dichromate, were mixed with the lyophilized IBV vaccine reconstituted with 10 mL of sterile deionized water and poured into a 100 mL beaker. The coccidiosis vaccine was then poured into the 100 mL beaker containing the IBV vaccine. This mixture was gently swirled and allowed to sit at room temperature for 1 minute. This mixture was then poured into a 4 L Erlenmeyer flask containing 2.1 L of sterile deionized water and stored the same way as the previous experiment.

Samples from each group were taken as described in Experiment 1.

IBV vaccine titrations. IBV virus from collected samples was titrated using a conventional method (8). Briefly, 10-fold serial dilutions of each vaccine group were made in phosphate-buffered saline and inoculated into five 9-10 day old embryonated SPF eggs per dilution (0.1 mL/egg). Inoculated eggs were incubated at 37°C for 7 days and candled each day for embryo mortality. Inoculated eggs were opened to observe any IBV-specific lesions at 7 days post inoculation. Embryo death within 24 hrs of inoculation was not counted and was not used in titer calculations. Virus titers were calculated using the method by Reed and Muench (9) and expressed as the 50% embryo infectious dose (EID50).

RESULTS

Coccidiosis vaccines that do not contain potassium dichromate (Coccivac B52, Advent, and InOvoCox) did not influence IBV vaccine titer in this experiment. There was no significant reduction in titer between groups over time (Figure 1A) or when comparing the mean titer for the time course experiment (Figure 1B). The Advent and InOvoCox vaccine mixtures with IBV both showed a slight reduction in mean IBV titer of 0.1 log EID_{50}, while the Coccivac B52 mixture with IBV showed a slight increase in mean IBV titer of 0.3 log EID_{50} (Figure 1B). Similarly, there was not a significant reduction in IBV titer at any specific time point during the experiment for the Hatchpak Cocci III or the Immucox I and Ma5 mixtures (Figure 2), but there was a reduction in mean IBV vaccine titer for the experiment by 0.5 logs EID_{50} (Figure 2). The same loss of titer was seen when Hatchpak and Immucox vaccines were mixed with IBV vaccine in a concentrated form as well (Figure 3), indicating that the potassium dichromate may be having an immediate effect on the IBV vaccine that does not increase over time.

DISCUSSION

Being able to combine IBV and coccidiosis vaccines could improve the vaccination process in

several respects. First, it can alleviate problems with larger application volumes and possible over-wetting of chicks in the hatchery. Second, combining vaccines in an effective delivery will streamline the vaccination process and make the entire system more efficient, possibly allowing for more chicks to be processed during each shift. Third, it has recently been shown that applying IBV vaccines in larger volumes improves vaccine take (10). By combining the vaccines and applying them in the larger application volume recommended for coccidiosis vaccines, one can take advantage of this without increasing the total application volume. In the future, field studies using these combined vaccines in a hatchery needs to be examined to fully elucidate any effects that the vaccines may have on each other and any differences seen in protection in the field from disease challenge, though some producers are currently using this strategy.

The results of this study confirm that the potassium dichromate chemical found in some coccidiosis vaccines may negatively impact IBV vaccine titer. This has long been suspected, and is indicated on the coccidiosis vaccine label. While the reductions in mean IBV vaccine titer when mixed with coccidiosis vaccines containing dichromate were not dramatic (loss of 0.5 log EID_{50}), they were consistent between vaccine (Hatchpak Cocci III and Immucox I) and mixing method (diluted vs concentrated). When coupling this .5 log reduction in IBV vaccine titer with known inefficiencies in hatchery operation and spray application, it is likely that the minimum infectious dose of IBV vaccine needed to properly immunize chicks will not be reached. It must also be noted that the vaccine mixtures were kept refrigerated over the 4-hour course of the experiment, which is the ideal condition for maintaining longevity of IBV vaccine titer. In a hatchery setting, this would not always be the case and the reduction in viral titer may be more pronounced in applications where vaccine temperature is not controlled effectively.

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Figure 1. Comparison of Ma5 IBV vaccine titer with or without Coccivac B52, Advent, or InOvoCox coccidia vaccines in application volume dilutions.

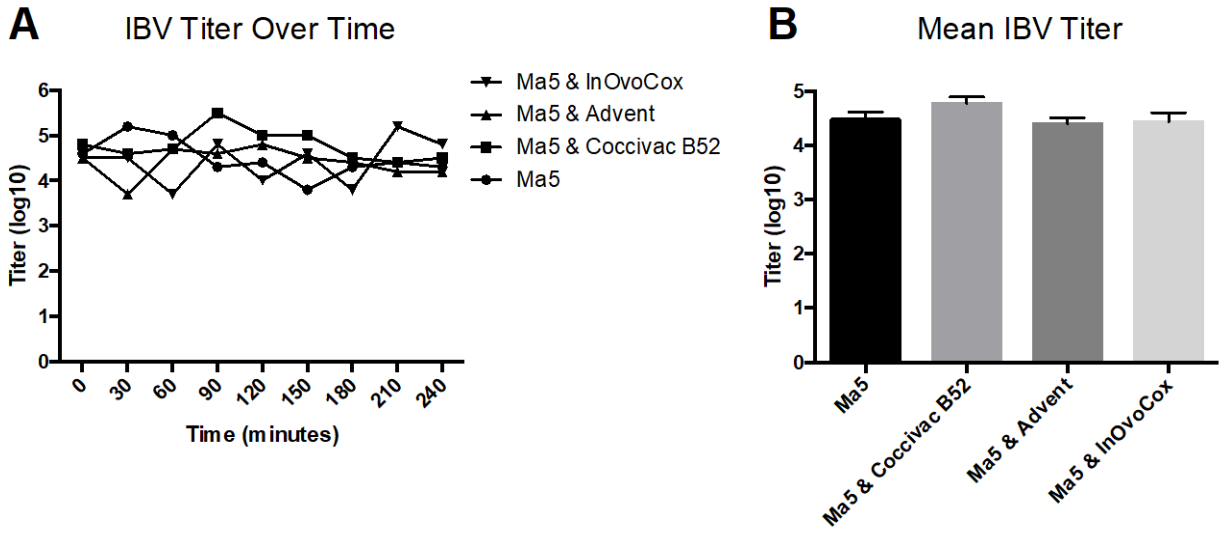


Figure 2. Comparison of Ma5 IBV vaccine titer with or without Hatchpak III or Immucox I coccidia vaccines in application volume dilutions.

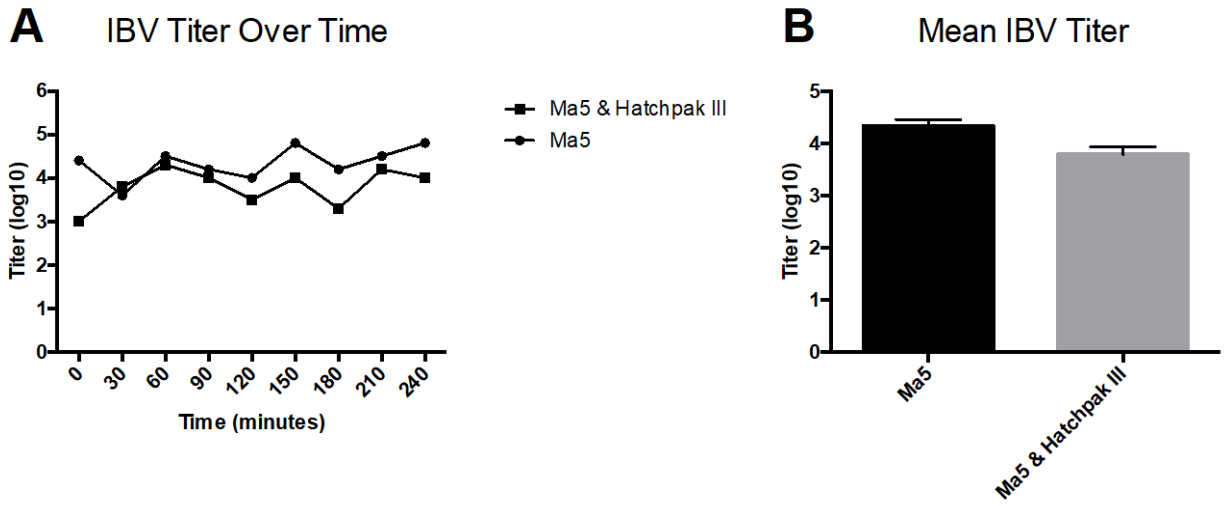
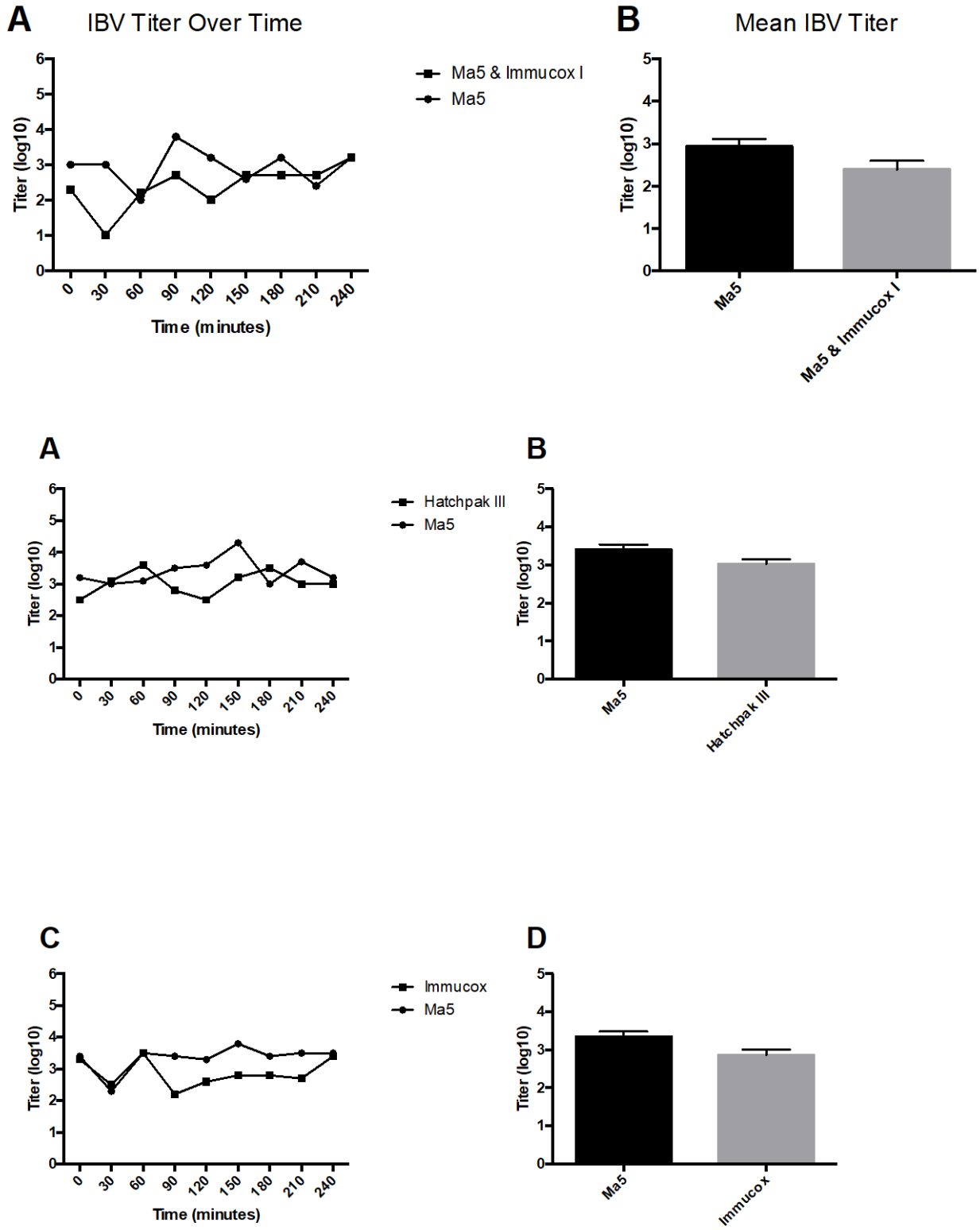


Figure 3. Comparison of Ma5 IBV vaccine titer with or without HatchPak III or Immucox I coccidia vaccines in a concentrated volume.



MOLECULAR DETECTION AND CHARACTERIZATION OF FADV IN MALAYSIA

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SUMMARY

In this study, poor chick quality and mortality was two times higher than usual. Post-mortem analysis showed hydropericardium, enlarged pale and friable liver. On-site PCR showed positive for FAdV infection and serotyping PCR showed those strains are closed to serotype 8b. In comparison to sequences with > 97% identity, the closest sequence was first reported in Hungary (97.27%) in 2009, and then mainly in China and Peru from 2013 to 2017. In 2019, Indonesia and Turkey also published sequences with > 98% identity. On-site FAdV detection enables veterinarian to provide appropriate actions and serotyping PCR provides information to identify against the endemic FAdV serotype for suitable vaccine type for breeder.

INTRODUCTION

Fowl adenoviruses (FAdVs) cause inclusion body hepatitis (IBH), gizzard erosions, and hepatitis-hydropericardium syndrome. IBH have been reported in different countries with mortality up to 30% (1). Twelve serotypes have been identified and cross-protection between serotypes is limited (2).

FAdV-2, 8a, 8b, and 11 are believed to be the agents for IBH outbreak (3). Vaccination and good biosecurity practices are needed to prevent disease outbreak with significant economic losses. Due to limited cross protection between serotypes, a matching vaccine serotype to fight against field strain is a critical issue in the field.

PCR can be a good candidate to aid in diagnosis of FAdV. On-site PCR detection allows veterinarians to identify the disease agent immediately. Following Hexon sequencing point out the serotype of endemic field strain, which is important for veterinarians to select the right vaccine.

In this report, two cases were reported to demonstrate how PCR can be a good tool to aid in diagnosis. With PCR results and sequencing information, veterinarians can provide precise diagnosis and vaccine type with scientific evidence.

MATERIALS AND METHODS

The suspected population were sampled and tested with POCKIT™ Central Fowl Adenovirus Premix Reagent (GeneReach). For sample with positive results, the Hexon sequenced were further amplified and sequenced. Phylogenetic analysis was conducted using MEGA version 6 (4).

RESULTS

There are two case reports in this study. In case 1, a broiler farm with 140,000 birds allocated in eight close house system. Suspected IBH & Reovirus infections were observed in previous batch. In this batch, high mortality started from day 18 until harvest with about 1.0-1.3% per day. Poor weight uniformity was observed (on day 14: low-340g; high-440g.) Necrotic enteritis and chronic respiratory disease started from day 28 until harvest and antibiotic treatments along with supportive treatments given from day 14 showed no significant improvement. FAdV were detected with on-site nucleic acid analyzer and sample were further sequenced and confirmed as serotype 8b (Figure 1).

In case 2, a broiler farm with 40,000 birds was suspected with IBH. Mortality started to spike at day 35, the first day of harvesting and after. Cumulated mortality from harvest to the end of harvesting was 5%. Post-mortem analysis showed congested liver, hydropericardium, and severe nephritis (eight of eight birds). FAdV were detected with on-site nucleic acid analyzer and sample were further sequenced and confirmed as serotype 8b (Figure 2).

CONCLUSIONS

In both case studies, FAdV was immediately diagnosed with on-site PCR system, which provided molecular evidence to aid in diagnosis with clinical signs. Both samples were further sequenced and phylogenetic analysis showed both samples were belonged to serotype 8b. With the evidence of FAdV infection and the serotype of FAdV endemic in the farm, the veterinarian can review the vaccination program in the breeder and optimize with appropriate vaccine to fight against the endemic field strain. On-site PCR detection can be a useful tool to aid in diagnosis in time and help veterinarians to provide corrective measures right away with scientific evidence.

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Figure 1. The phylogenetic analysis of sample (20-009) in case 1.

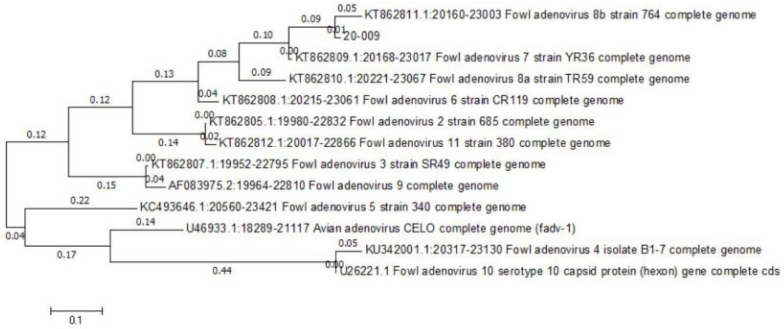
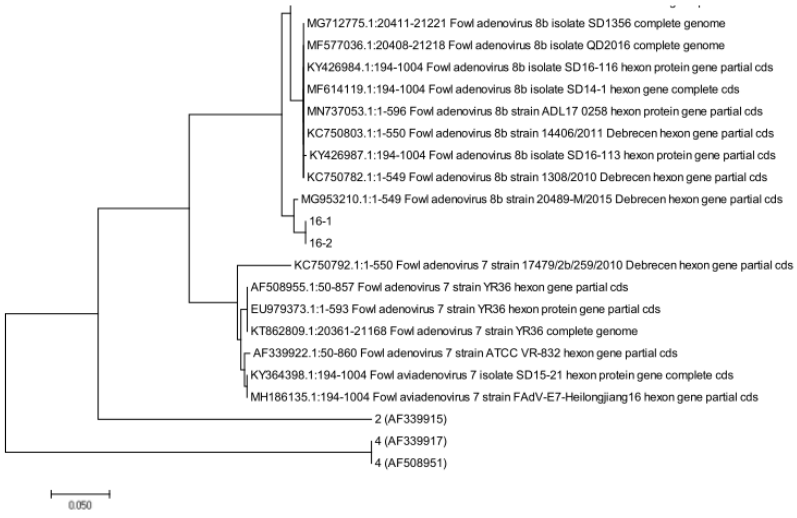


Table 2. The phylogenetic analysis of sample (16-1 & 16-2) in case 2.



HOW TO USE ANTIBIOTICS SMARTLY WITH ON-SITE PCR-CASE STUDIES

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SUMMARY

Mycoplasmosis is common around the world, which causes respiratory diseases, poor growth, down-grade of meat quality and results in economic losses in broiler industry. People rely on antibiotics for treatment in Asian countries, but reduction of antibiotics now becomes an important issue. Two case studies showed how to use antibiotics smartly with the help of on-site polymerase chain reaction (PCR) test in Asian region. In case 1, *Mycoplasma gallisepticum* (MG) was detected at the day of age, although tilmicosin was treated, insufficient dosage did not solve the problem and MG was still detected until day 28 despite several drugs were treated continuously. In case 2, MG and *Mycoplasma synoviae* (MS) were detected at the day of age. Sufficient doxycycline was treated immediately and mycoplasmosis turned negative at day 7 until harvesting. Using on-site PCR detection system to check the presence of MG and MS on day old chicks and confirm the treatment efficacy is a good strategy for next-generation mycoplasmosis control.

INTRODUCTION

Avian mycoplasmosis is a prevalent respiratory disease in the poultry farm. Day old chicks may be contaminated with MG and MS from vertical transmission route from breeders. Mycoplasmosis in poultry will cause varying degrees of respiratory distress, rales, difficulty breathing, coughing, sneezing, and nasal discharge. It will present low feed conversion rate, reducing feed efficiency and weight gains in the broiler. Moreover, it will cause down-grade in meat quality and result in the greatest economic loss than other diseases (1).

For disease control, farmers rely on antibiotics treatment in some countries (2). However, antimicrobial resistance becomes serious and post threats to public health (3). Reduction of antibiotics becomes an important issue. The abuse of antibiotics still goes on in many Asian countries and a proper strategy for the reduction of antibiotics is needed urgently.

PCR can be a good candidate to help the optimization of antibiotics usage. Instead of time-consuming culture, PCR provides the possibility for *Mycoplasma* timely detection (4). Once we got positive results of MG/MS, chickens can be treated with antibiotics precisely and immediately with scientific evidence. PCR can also be a good tool for treatment evaluation.

In this report, two distinct cases were reported to show how PCR can be a good tool to use antibiotics smartly. Both on-site PCR for timely detection and antibiotics treatment with sufficient dose are important for the reduction of antibiotics.

MATERIALS AND METHODS

For each check point, chickens were random selected from a house and trachea swab were collected individually. The sample for day old chicks were collected upon arrival at the farm. In case 1, test was conducted in a house with 30,000 flocks. In case 2, test was conducted in three houses with 25,000 flocks each. Five swabs were in a pool and placed into a 15 mL tube with 3 mL taco™ Sample Storage Solution (GeneReach). MG and MS were tested with POCKIT™ Central Nucleic Acid Analyzer following the instructions for use to check the presence of *Mycoplasma*.

RESULT

In case 1, MG test was positive for day old chicks while MS was negative. Chickens treated with Tilmicosin through drinking water immediately for 3 days, but insufficient dose (0.08 mg/L, only 1% from recommendation) which was not effective and MG tests were still positive at day 7. Several antibiotics were treated continuously but did not work until day 28 before harvesting (Table 1).

In case 2, MG test was positive in two houses and MS test was positive in three houses for day old chick. Chickens treated with sufficient doxycycline in feed and MG/MS tests were negative at day 7 and until day 28 before harvesting (Table 2).

CONCLUSION

In both case studies, *Mycoplasma* was detected from day old chick. This result revealed potential vertical transmission of *Mycoplasma* from parent stock. Although MG/MS vaccination were applied in parent stock in certain countries, vaccination only prevents

severe clinical symptoms but not guarantees free of infection.

Although *Mycoplasma* was detected early at the day of age, following treatments were different in two case studies. Treatment with insufficient dose did not solve the problems but increase the cost of antibiotics. This flock may have very resistance MG strains. Instead, on-site PCR detection and treatment with sufficient antibiotics at earliest helps farmer to identify the mycoplasmosis problem. Besides, PCR was potentially helpful to solve the mycoplasmosis problems from parent stocks since it pointed out the potential vertical transmission routes for mycoplasmosis.

For the reduction of antibiotics, a suitable method for early detection and treatment evaluation is very important. On-site PCR provides evidence for the presence of *Mycoplasma*. What's more exciting is that PCR can also be a good tool to evaluate the efficacy of antibiotics treatment. For next-generation mycoplasmosis control in modern farm, on-site PCR can help farmer and veterinarian to use antibiotics smartly.

ACKNOWLEDGEMENT

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Table 1. The PCR result and the strategies for antibiotics treatment in case 1.

Time course/PCR result	Day Old Chicks	Day 7	Day 14	Day 21	Day 28
MG*	1/2	2/2	2/2	2/2	2/2
MS*	0/2	0/2	0/2	0/2	0/2
Treatment					
Day 1-3	Tilmicosin phosphate 0.08 mg/L** (low dosage)				
Day 10-14	Tylosin Tartrate 50 mg/L, Doxycycline HCl 100 mg/L, cholistin sulphate 0.025 MIU/L, Bromohexine HCl 2.5 mg/L, Streptomycin Sulphate 10 mg/L				
Day 20-23	Avilosin 35 g/1000 kg body weight				
Day 28-30	Amoxicillin trihydrate Eq. to Amoxicillin base 7.5 mg/L, cholistine sulphate 0.025 MIU/L				

*: The results are showed as (positive/number of tests)

**: The dosage is far from medication dose. The water consumption per day for day old chicks is around 80-100 mL, which equivalent to 0.008 mg Tilmicosin phosphate. However, 0.75 mg is needed for treatment.

Table 2. The PCR result and the strategies for antibiotics treatment in case 2.

Time course/PCR result		DoC	Day 7	Day 14	Day 21	Day 28
House 1	MG	-	-	-	-	-
	MS	+	-	-	-	-
House 2	MG	+	-	-	-	-
	MS	+	-	-	-	-
House 3	MG	+	-	-	-	-
	MS	+	-	-	-	-
Treatment						
Day 1-3	Doxycycline 1kg/ton feed					

A MICROBIAL PHYSIOLOGY APPROACH TO THE DETECTION AND ENUMERATION OF *CLOSTRIDIUM PERFRINGENS* IN THE POULTRY HOUSE ENVIRONMENT

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SUMMARY

Necrotic enteritis (NE) is a significant intestinal disorder of poultry caused by the spore-forming, obligate anaerobe, *Clostridium perfringens*. Antimicrobials have historically prevented NE until concerns over antibiotic resistance resulted in pressures to limit use in poultry, causing NE reemergence. *C. perfringens* spores persist in the environment, including poultry farms and potentially cause significant disease. Our central hypothesis is avian pathogenic *C. perfringens* strains produce high spore loads on some farms, predisposing birds to NE by spore ingestion from a heavily contaminated environment. Capitalizing on *C. perfringens* physiology and metabolism, we have developed a media formulation that "poisons" other poultry environmental bacteria using an oxidizing agent coupled with a physical heat treatment to determine spore abundance. The medium was formulated to be selective and differential for *C. perfringens* spores compared to other species in poultry litter that cannot survive conditions where nitrate reductase activity produces cytotoxic chlorite from a chlorate source, such as *C. perfringens* can. Lecithinase activity by *C. perfringens* is visible on egg yolk, a lecithin source that forms distinct white zones around the colonies. Coupled with a heat treatment to eliminate vegetative cell growth, spore isolation from poultry litter was achieved. Using our medium formulation and heat treatment, we identified lecithinase-positive colonies from the poultry house environment and subsequently confirmed them as *C. perfringens* by MALDI-TOF. We specifically found *C. perfringens* spores in the environment of poultry farms with a history of NE compared to farm without NE. The ability to monitor *C. perfringens* spore abundance is a game changer to understanding NE on poultry farms.

INTRODUCTION

NE is one of the most challenging intestinal bacterial diseases in modern poultry production and is a significant cause of production losses from decreased feed conversion and mortality. NE is caused by the spore-forming, obligate anaerobe, *Clostridium perfringens* that regularly colonizes the gastrointestinal tract, and poultry litter may be a reservoir (1). Antimicrobials have historically prevented NE until concerns over antibiotic resistance resulted in pressures to limit its use in poultry, causing NE reemergence with no preventative strategies in place. The spore-forming capability of *C. perfringens* protects the microbe from treatments used on poultry litter, resulting in spore germination when ingested by the birds and the onset of disease (2). The avian pathogenic *C. perfringens* produce several types of toxins, commonly including the α-toxin, but NetB and β2 toxin are associated with increased mortality in flocks (3, 4). Because the *C. perfringens* are capable of producing spores in the environment, we are interested in determining whether poultry houses with a history of NE contain a higher spore load of *C. perfringens*.

The presence of Clostridiaceae in the poultry environment is relatively minor to other species according to 16s genome sequencing of litter microbial ecology studies with an abundance of only ~3% (5). Therefore, a differential and selective culture medium coupled with a heat treatment was developed using the physiology of *C. perfringens* to select media components to inhibit other poultry environmental bacteria to enable detection and quantitation of pathogenic isolates. The heat treatment isolates spores for enumeration from poultry litter and the selective, differential media enriches for pathogenic *C. perfringens*. According to Sacks and Thompson, if spores are subjected to longer time in a heat treatment than it takes to kill the vegetative cells, proteins in the spore membrane are broken down and germination is more efficient (6). The lecithinase activity (α-toxin) of *C. perfringens* is not present in most microbes in the poultry litter environment, resulting in activity that is differential when used in agar that includes lecithin. The aerobic, respiratory bacteria in poultry litter express nitrate reductase activity when a nitrate source is present and oxygen is absent. When nitrate reductase is coupled with a chlorate source as an oxidizing agent, the nitrate reductase activity metabolizes chlorate into cytotoxic chlorite, poisoning

aerobic bacteria. Understanding the physiology of *C. perfringens* compared to other poultry environmental bacteria allows for a better understand of the importance of the environment to NE outbreaks.

MATERIALS AND METHODS

Poultry litter samples were collected from various areas in five houses (Houses 1-5). Of the houses collected, Houses 1-3 contained birds diagnosed with NE, House 4 had a recurring history but was not currently suspected of an outbreak, and House 5 had no history of NE and was not suspected of outbreak. Litter was collected from several spots throughout the poultry house and placed in Whirl-paks stored at -20℃. To isolate the bacteria from the litter, 1 gram of litter was weighed from each bag, suspended in phosphate buffer saline in a wrist-action shaker, inserted into a filtration spin-column, washed with Tween80 buffer, and centrifuged to collect bacterial cells isolated from the dirt and debris. Bacteria pellets were resuspended in a freezer stock medium and stored at -80℃. A *C. perfringens* control, SM101, was obtained from Dr. Stephen Melville at Virginia Tech that readily sporulates (7), providing insight into *C. perfringens* sporulation and germination efficiency to compare to wild-type strains in the poultry litter environment. Before the experiment took place, *C. perfringens* was incubated in Duncan-Strong Sporulation medium with an inclusion of caffeine for 48 hours, as described by Sacks and Thompson (8). *Salmonella enterica* Typhimurium LT2 was a negative control for sporulation and anaerobic respiratory growth.

Bacterial pellets in freezer stock were serially diluted and plated on tryptic soy agar (Sigma-Aldrich, St. Louis, MO) to determine total aerobic counts and MacConkey (Thermo Fisher Scientific, Waltham, MA) to determine total Gram-negative enteric counts. In order to identify conditions to prevent anaerobic respiration, potassium chlorate inclusion in tryptic soy agar was screened at multiple concentrations and incubated aerobically and anaerobically. Potassium nitrate was included to provide the nitrate source for the proposed nitrate reductase activity along with sodium molybdate and sodium selenite to induce activity further. Glucose was either included or excluded to determine if the presence would allow bacteria in the anaerobic environment to perform anaerobic respiration. The anaerobic incubation would force bacteria that perform anaerobic respiration into a metabolic pathway that inhibited growth by the media. A heat treatment was integrated to eliminate vegetative cells (6).

Because the α-toxin production of *C. perfringens* has lecithinase activity, egg yolks were incorporated into the selective medium to make it differential, as well. The lecithin present in the yolks would cause a white zone to form around the *C. perfringens* colonies, making them visibly distinct. Colonies with a “halo” were isolated and sent to the Veterinary Diagnostic Lab at Virginia Tech to be identified using mass spectrometry proteomics (MALDI-TOF).

RESULTS

Total aerobic counts on TSA and total gram negative enterics on MacConkey were not different among houses suspected of an NE outbreak ($P > 0.05$) as shown in Table 1. Inclusion of potassium chlorate at 0.1%, 0.2%, and 0.5% in TSA did not inhibit growth of poultry litter bacteria. An inclusion of 1.0% eliminated growth of LT2 while allowing C. perfringens growth ($P < 0.05$). Once proper potassium chlorate inclusion was determined, heat treatment eliminated LT2 after 5, 10, and 15 minutes at 75℃, but after 20 minutes of the heat treatment, *C. perfringens* showed greater growth on TSA due to breakdown of proteins from longer heat exposure. Spore enrichment using Duncan-Strong sporulation medium and Duncan-Strong Improved sporulation medium both made the *C. perfringens* sporulate, and there was no difference between the two using the control strain ($P > 0.05$). The addition of egg yolk into the reinforced clostridial agar identified colonies that exhibited halos indicating presumptive *C. perfringens*. Once medium was configured to include 1.0% potassium chlorate and heat treatment was determined to be 20 minutes at 75℃, Houses 1-5 gave insight into spore load in houses with varying suspicions of NE. Anaerobe and strict anaerobe vegetative cells were not different among poultry houses. However, there were fewer lecithinase positive colonies on the egg yolk agar with potassium chlorate than the egg yolk agar base. *Salmonella* LT2 did not grow before heat treatment on the selective medium nor after the heat treatment while the control *C. perfringens* remained viable. Spores of both the anaerobes and strict anaerobes grew on both the selective and the non-selective medium in Houses 1-4, but House 5, which had no history and no suspicion of an NE outbreak, did not yield *C. perfringens* spores within the limits of detection. Most of the spores that grew on both mediums were also lecithinase positive and suspected to be *C. perfringens*. The MALDI-TOF identified these colonies from Houses 1-4 as *C. perfringens* and justifying the isolation of *C. perfringens* spores from poultry litter using our medium and heat treatment.

DISCUSSION

In this study, differential and selective medium was formulated using microbial physiology that separates *C. perfringens* spores from other poultry litter bacteria. Coupled with a heat treatment, the overall method would enrich for pathogenic *C. perfringens* spores. Application of the method to farms with a history of NE outbreaks revealed that *C. perfringens* spores were present in litter in a higher abundance than a farm with no NE. The ability to monitor abundance of *C. perfringens* in a poultry litter environment could be paramount to production farms to have a fast method to diagnose flocks with NE, rather than experiencing the mortality spike with no confirmation of the disease, and to better identify flock houses with an environmental spore load leading to repeated outbreaks. Using our proposed method, researchers and producers have the ability monitor abundance in a similar fashion to the common practice of detecting *Salmonella* in poultry houses, thus improving economics for the producers and animal welfare.

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Table 1. Litter microbiota composition and enumeration of *Clostridium perfringens* for poultry farms with and without necrotic enteritis.

Litter Bacteria	*C. perfringens*[+]	*Salmonella*	House 1[7] NE+	House 2[7] NE+	House 3[7] NE+	House 4[7] Recurring NE+	House 5[9] NE-
Total Aerobic Bacteria[1]			8.80	8.48	9.15	8.90	8.12
Total Gram Negative Enterics[2]			4.97	3.12	3.67	5.04	3.48
Anaerobe-Vegetative Cells[3]	8.35	>9.00	4.75	6.27	5.90	6.20	5.92
α toxin-positive (lecithinase)[6]			4.33	3.79	3.78	4.82	5.60
Strict Anaerobe-Vegetative Cells[4]	8.30	<3.00	6.61	7.30	7.20	6.00	5.99
α toxin-positive (lecithinase)[6]			3.88	6.22	<3.00	5.82	<3.00
Anaerobe-Spores[5]	6.48	<3.00	3.83	4.22	3.98	3.70	<3.00
α toxin-positive (lecithinase)[6]			3.83[10]	4.22[10]	3.98[10]	3.34[10]	<3.00
Strict Anaerobe-Spores[5]	4.88	<3.00	3.60	3.74	3.90	3.48	<3.00
α toxin-positive (lecithinase)[6]			3.60[10]	3.74[10]	3.30[10]	3.4[10]	<3.00

[1]

[1]Tryptic Soy Agar (TSA), [2]MacConkey agar. Plates were incubated aerobically at 37°C, overnight; [3]Reinforced Clostridial Agar with egg yolk. Plates were incubated anaerobically at 37°C for 48 hours. [4]Reinforced Clostridial Agar with egg yolk and potassium chlorate. [5]Samples were heat treated 75°C for 20 minutes before plating on Reinforced Clostridial Agar with egg yolk with/without potassium chlorate. [6]Lecithinase-positive colonies that are potentially α-toxin positive. [7]House with necrotic enteritis break; [8]Reocurring history of necrotic enteritis; [9]No history of necrotic enteritis; [10]Identified as *C. perfringens* by MALDI TOF. Values are in Log10 form.

DEVELOPMENT OF RECOMBINANT PICHINDE VIRUS VECTORED VACCINE AGAINST TURKEY ARTHRITIS REOVIRUS

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SUMMARY

Vaccination may be an effective way to reduce turkey arthritis reovirus (TARV) induced lameness in turkey flocks. However, there are currently no commercial vaccines available against TARV infection. Here, we describe the use of reverse genetics technology to generate a recombinant Pichinde virus (PICV) that expresses the Sigma C and/or Sigma B proteins of TARV as antigens. Nine recombinant TARV-PICVs were developed carrying the wild type S1 and/or S3 genes from three different TARV strains. In addition, three recombinant TARV-PICVs were produced carrying codon optimized S1 and/or S3 genes of a TARV strain. The S1 and S3 genes and antigens were found to be expressed in virus-infected cells via reverse transcriptase-polymerase chain reaction (RT-PCR) and direct fluorescent antibody (FA) technique, respectively. Turkey poults inoculated with the recombinant TARV-PICV vaccine expressing the bivalent TARV S1 and S3 antigens developed high anti-TARV antibody titers indicating the immunogenicity (and safety) of this vaccine. Future *in vivo* challenge studies using a turkey reovirus infection model will determine the optimum dose and protective efficacy of this recombinant virus-vectored candidate vaccine.

INTRODUCTION

TARVs re-emerged in 2011 in Minnesota and other states in the US (1-3). The viral genome consists of 10 segments of dsRNA grouped into large (L1, L2, L3), medium (M1, M2, M3), and small (S1, S2, S3, S4) segments based on migration pattern on polyacrylamide gel electrophoresis (4, 5). The genome has 12 open reading frames (ORFs), which encode for eight structural and four non-structural proteins. The proteins encoded by L, M and S genes are lambda (λ), mu (m) and sigma (σ), respectively (5). The S1 and S3 segments translate into σC (cell attachment) and σB (outer capsid) proteins, respectively. The σC protein is a minor outer capsid protein and has been identified as cell attachment protein responsible for virus entry in the host cell (6). In addition, σC possesses both type and broad-specific epitopes and elicits reovirus-specific neutralizing antibodies in the host (7). The protein σB is a major component of the viral outer capsid that contains group-specific neutralizing epitope (8). Several reports on subunit vaccines for chicken reovirus (CARV) are available which express either partial fragment of σC protein (9) or full length σC protein alone (10) or in combination with σB protein (11). The recombinant σC proteins as subunit vaccines has been reported against duck reovirus (12). Since TARVs are similar to CARVs in terms of the genomic structure, segment sizes and open reading frames, the σC and σB of TARV should be useful in the development of uni- or multi-valent virus vectored vaccine against TARV.

TARV-infected turkeys display clinical lameness due to tenosynovitis and arthritis resulting in huge economic losses mainly due to culling. No commercial vaccine is available to protect turkey flocks from the emerging TARV strains. Some turkey producers rely on the use of autogenous vaccines. However, the evolving nature of the virus creating new divergent strains poses a challenge for regular update of the vaccines. Additionally, there is no live vaccine available that can be used for priming before boosting breeder turkeys with injectable killed vaccines.

Using a live and safe vectored vaccine to deliver TARV antigens is a reasonable alternative to live reovirus vaccines. Recently, a PICV vector was

developed that safely and effectively delivered the model antigens, the influenza viral hemagglutinin (HA) and nucleoprotein (NP) (13). This arenavirus was first isolated from its natural host *Oryzomys albigularis* (rice rats) in the Pichinde valley of Colombia, South America (14). Arenaviruses are enveloped RNA viruses with a bi-segmented genome and are known to target dendritic cells and macrophages at early stages of infection, making it a potentially powerful vaccine vector (15-18).

Using reverse genetics technique, a live recombinant PICV (strain 18) with a tri-segmented RNA genome (rP18tri) has been developed to carry and express up to two foreign genes. One of the foreign genes could be the green fluorescent protein (GFP) that can be used to mark virus-infected cells in cell culture. The rP18tri has been shown to be attenuated both in vitro and in vivo and has the ability to induce cell-mediated and humoral immune responses. For example, mice immunized with recombinant PICV-hemagglutinin (PICV-HA), expressing the modeled influenza hemagglutinin protein, developed a strong humoral response against HA that afforded complete protection against a lethal avian influenza virus infection (13).

The present study was undertaken to develop recombinant PICV vaccines expressing one or both TARV antigenic Sigma C (σC) and Sigma B (σB) proteins. In addition, the vaccines were administered to turkey poults to determine its safety and efficacy. This is a 'proof of concept' study for the development and recovery of recombinant PICV-TARV viruses and testing the safety and immunogenic properties of the turkey reovirus σC and σB protein(s) *in vivo*.

MATERIALS AND METHODS

Cells and viruses. QT-35 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and 50 ug/mL penicillin-streptomycin. Baby hamster kidney (BHK-21) cells, BSRT7-5 cells (BHK-21 cells stably expressing T7 RNA polymerase) were grown in Eagle's minimal essential medium (MEM) and LMH cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Sigma-Aldrich) that contained 10% FBS, 1 ug/mL Gentamicin, and 50 ug/mL penicillin-streptomycin. Media for BSRT7-5 cells was also supplemented with 1 µg/mL geneticin (Invitrogen-Life Technologies). Three strains of turkey arthritis reovirus (SKM73, SKM95 and SKM121) isolated in QT-35 cells from tendons of lame turkeys were used. These viruses were selected based on their pathogenicity and genomic characterization.

Pichinde virus plasmids. Three plasmids were used:

(i) pP18S1-GPC/MCS encodes the glycoprotein GPC and a multiple-cloning-site (MCS) to clone the gene of interest.

(ii) pP18S2-MCS /NP encodes the nucleoprotein NP and an MCS.

(iii) pP18L plasmid (the L plasmid) expresses the full-length antigenomic strand of the rP18L segment under the control of T7 promoter and does not contain any specific site to clone foreign genes (13).

Preparation of vectors and gene inserts. Genomic RNA was isolated from three TARV isolates (SKM73, SKM95, and SKM121). Full-length open reading frame (ORF) of S1 and S3 genomic segments of these viruses were amplified. Additionally, the S1 and S3 ORF sequences of SKM121 were codon-optimized and commercially custom synthesized in a pUC vector. The cDNA was synthesized using random primers and SuperScript™ IV First-Strand Synthesis (Thermo Fisher Scientific, Catalog#18091200, Waltham, MA, USA) and was PCR amplified using specific cloning primers and Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB Catalog#M0531S, Ipswich, MA, USA). The NheI and Kozak sequences were added in the forward primer so that these features are included in the amplified product. Similarly, XhoI and sequence tags (FLAG tag in S1 and HA tag in S3 gene) were added in the reverse primer. The reaction conditions were: 98ºC for 30 sec (initial denaturation); 5 cycles of denaturation at 98ºC for 10 sec, annealing at 66ºC for 30 sec and extension at 72ºC for 1 min; 30 cycles of denaturation 98ºC for 10 sec, annealing at 72ºC for 30 sec and extension at 72ºC for 1 min; final extension at 72ºC for 7 min and 4ºC hold. The PCR amplified products (S1 and S3 genes of all three isolates) and the plasmids of the PICV (pP18S1-GPC/MCS and pP18S2-MCS /NP) were restriction digested (NheI and XhoI, NEB) and gel purified using QIAquick gel extraction kit (Qiagen Catalog#28704, Germantown, MD, USA). The codon-optimized versions of ORF were extracted from the pUC vector by restriction double digestion.

Cloning and transfection. The S1 and S3 gene inserts were ligated in MCS region of plasmids pP18S2-MCS/NP and pP18S1-GPC/MCS, respectively, using 5 U/µL of T4 DNA ligase (ThermoFisher Scientific, Catalog#EL0011). The ligation reaction mix was used to transform competent bacterial cells (DH5α) followed by selection using ampicillin antibiotic. All plasmids (plasmid pP18L, pP18S1-GPC/GFP, pP18S2-GFP/NP, and recombinant plasmids pP18S1-GPC/S3 and pP18S2-

S1/NP) were isolated by plasmid midi prep kit (Sigma-Aldrich). Recombinant plasmids were PCR-confirmed for reovirus genes and sequence-confirmed for correct orientation and reading frame. The recombinant plasmids were used to transfect BSRT7-5 cells in various combinations (Table 1) using Lipofectamine™ 3000 transfection reagent (ThermoFisher, Catalog#L3000008) following manufacturer's instruction with minor modifications. After 48, 72 and 96 h post transfection, cell supernatants were collected and stored at -80°C. Different monovalent and bivalent vector viruses were generated as detailed in Table 1. The resultant viral recovery was confirmed by observing green fluorescence of GFP in inoculated cell culture. The rescued virus was then grown in BHK-21 cells and the GFP green fluorescence was observed. The presence of reovirus genes was confirmed by gene-specific PCR.

Detection of reovirus antigenic proteins. The expression of σC and σB proteins by recombinant tri-segmented PICV vaccine viruses in BHK-21 cells was verified by direct fluorescence antibody (FA) assay. The BHK-21 cells were inoculated with the recombinant PICVs and at 96 h post infection (hpi), the cells were plated on 12-chamber slides, dried and fixed in acetone followed by addition of polyclonal FITC-conjugated anti-avian reovirus antibodies (National Veterinary Services Laboratory, Ames, IA, USA, Reagent#680-ADV). After incubation at 37ºC in a CO_2 incubator for 2 hours, counter staining was done using 0.1% Evan's blue biological stain (EBBS). The slides were then mounted and examined under a fluorescent microscope to observe FITC green fluorescence, which was indicative of avian reovirus protein expression by the vaccine viruses.

Vaccination experiment. Four groups of turkey poults (five birds/group) were inoculated with 0.3 mL of the different recombinant PICVs containing codon optimized gene segments of TARV-SKM121 (monovalent PICV-S1, monovalent PICV-S3, bivalent PICV-S1/S3, and PICV-control without any TARV segment insertion) having $3x10^5$ PFU/mL via oral route at one week of age. Birds in all groups were given booster dose (0.3 mL, $3x10^5$ PFU/mL) with respective vaccine viruses at three weeks of age via intranasal (I/N) route. Blood samples were collected at three and five weeks of age. The birds were examined daily for any overt clinical signs or mortality. Birds displaying signs of severe illness were euthanized according to the guidelines laid by IACUC and research animal resources (RAR), University of Minnesota. At the end of the experiment, all birds were euthanized, and necropsy was done to detect the development of any gross lesions.

Serum neutralization assay. Sera were separated from the collected blood samples and subjected to serum neutralization assay against virus strain TARV-SKM121. Briefly, 4-fold serial dilutions of heat inactivated serum samples and 25 µL of reovirus preparation (100 $TCID_{50}$) were used. The virus-sera mixture was incubated at 37 °C for 1 hour before adding onto freshly seeded LMH (5×10^5 cell/well) with 10% fetal calf serum and incubated for four to five days. The plates were observed daily for the appearance of cytopathic effects (CPE). When CPEs was observed, the medium was removed, and the cells were stained with a 1% crystal violet solution prepared in 10% buffered formalin. The titer was recorded as the reciprocal of the highest dilution of serum that inhibited at least 50% cell destruction. Serum neutralization titers among different groups were subjected to statistical analysis using non-parametric Kruskal Wallis test followed by Mann Whitney U test for testing statistical significance at $P<0.05$.

RESULTS

Cloning of reovirus genes into PICV plasmids. The RT-PCR amplification of S1 and S3 ORF yielded the expected product sizes of 1031 bp and 1157 bp, respectively. These products were gel purified and cloned into pP18S2-MCS /NP and pP18S1-GPC/MCS, respectively. Restriction enzyme double digestion confirmed the presence of reovirus genes in recombinant PICV plasmids. Sanger sequencing confirmed the absence of mutation in cloned viral gene as well as their correct reading frame and correct orientation in the vector backbones (data not shown).

Plasmid transfection and virus rescue. Viable recombinant PICVs were rescued successfully following transfection of BSRT7-5 cells with the three plasmids in various combinations as shown in Table 1. The GFP expression was observed 48-72 h post transfection in cells transfected with at least one GFP-containing plasmid (all monovalent vaccines in Table 1). The GFP-expressing foci increased in size over the time course of transfection. The supernatants were collected from transfected BSRT7-5 cells and were used to infect BHK21 cells. Strong GFP expression was detected in infected BHK21 cells at 24-48 hpi using fluorescence microscopy indicating the rescue of viable viruses. At every rescue attempt, we obtained infectious viruses at 48-72 h post transfection. The recombinant viruses showed minor GFP fluorescence in QT-35 and LMH cells at 96 hpi. As expected, the bivalent viruses having two TARV genes on both plasmids did not produce any green fluorescence.

Recombinant PICVs expressing reovirus antigens. Strong GFP expression by infected BHK21

cells indicated successful rescue of recombinant viruses. The supernatant from infected BHK21 cells (passage P1, P2 and up to P3) was used to detect reovirus genes by RT-PCR. The results confirmed the presence of both viral genes in bivalent vaccine viruses and either S1 or S3 gene in the monovalent vaccine viruses. Direct FA was done to verify the expression of reovirus antigenic proteins (σC and σB) by the recombinant PICVs containing either S1 or S3 or both which showed varying degrees of fluorescence. Although we did not quantify the amount of fluorescence, PICVs containing SKM121 gene segments showed a remarkably higher degree of fluorescence, particularly the bivalent PICV that contained codon optimized S1 and S3 segments. Minimal fluorescence was observed in negative controls (cells that contained recovered PICV vector without any TARV segment).

Clinical signs and necropsy. Birds inoculated with the vaccines (monovalent and bivalent) neither displayed any clinical disease or illness during the study nor showed any gross lesions at necropsy.

Serum neutralization. At three weeks of age, three of four sera from birds inoculated with monovalent PICV-S3 vaccine and four of five sera from birds inoculated with bivalent PICV-S1/S3 vaccine showed serum neutralizing (SN) antibody titers of 64, which were significantly higher ($p<0.05$) than the other two groups (Fig. 1). At five weeks of age, only one of three birds inoculated with monovalent PICV-S1, monovalent PICV-S3 and PICV-control had an SN antibody titer of 64 while all five sera from birds inoculated with the bivalent vaccine had high SN antibody titers ranging from 64 to 256, which were significantly higher ($p<0.05$) than the other three groups (Fig. 4). Individual bird data shows that there was a remarkable increase in the SN antibody titers of birds inoculated with the bivalent PICV-S1/S3 vaccine at five weeks of age than the other groups (Fig.1).

DISCUSSION

Using PICV as a vector to express TARV antigenic proteins appears an attractive alternative to the use of live attenuated vaccines that may result in the emergence of mutant strains. Recombinant PICV expressing the hemagglutinin and nucleoprotein genes of influenza initiated humoral and cell-mediated immune responses and provided full protection against lethal influenza in mice [13] and was shown to be safe and effective in chickens (data not shown). Several subunit vaccines reported against chicken reovirus and duck reovirus involves expression of σC protein alone or in combination with σB proteins (9-12). The purpose of this study was to develop recombinant PICV-TARV vaccines that can carry S1 and/or S3 genes of turkey arthritis reoviruses. We successfully recovered PICV after insertion of TARV genomic segments in the PICV plasmids, detected the inserted genes and confirmed the expression of recombinant antigenic proteins.

A total of 12 different recombinant PICVs were developed. We used the wild type genes from three different TARV isolates in addition to codon-optimized genes from one TARV isolate in an effort to find an optimum TARV candidate to be used in developing a vaccine. The recovered PICV recombinants grew well in BHK-21 cells but showed minimal growth on QT-35 and LMH cells as determined by the expression of GFP in recombinant PICV that contained one each of TARV gene and GFP gene. No GFP gene was inserted in the bivalent recombinant PICV (containing both S1 and S3 genes of TARV) and hence they could not be subjected to the green fluorescence test. We assume that these viruses grew the same way as the monovalent viruses because their plasmids and transfection were done under the same conditions. The detection of the inserted genes and protein expression was helpful in determining successful rescue and growth of bivalent PICV. The use of RT-PCR to detect the inserted TARV genome in recovered viruses helped in determining the success of recovering recombinant PICV that contained either S1 or S3, or both (Please see supplementary figure of RT-PCR).

To confirm the expression of reoviral antigenic proteins by the recombinant PICVs, we used the direct FA technique using FITC-conjugated anti-avian reovirus antibodies. The FA procedure included dehydration and long fixation with acetone. These two steps helped eliminate GFP fluorescence and increased permeabilization of the cell membrane. The permeabilization of the cell membrane enabled the entrance of the FITC-conjugated antibody to the intracellular recombinant viral proteins. Although we did not have a quantitative method to measure the amount of the expressed protein, we could subjectively observe that the fluorescence produced by viruses recombined with SKM121 genes was more than that produced by SKM73 and SKM95. The bivalent PICV that contained codon optimized S1 and S3 of SKM121 showed the best fluorescence indicating the growth of these viruses, which did not have any GFP fluorescence after transfection.

Since codon-optimized PICV-SKM121 vaccines showed the best expression of S1 and/or S3 proteins of TARV, we used these vaccines for a pilot *in vivo* experiment. PICV vaccine without an insert of TARV gene was used as a control. The *in vivo* experiment was to determine the safety and humoral response generated by the recombinant vaccines in turkey

poults. Turkey poults were vaccinated with the same dose of vaccine (0.3 mL, $3x10^5$ PFU/ml) by oral and intranasal routes at an interval of two weeks (one week prime; three week booster). Poults were primed at one week of age because birds are susceptible to avian reovirus infection in the early days of their life (19, 20), hence vaccination strategies are designed to provide passive immunity from maternal antibodies by vaccinating breeders or by actively immunizing young bids with a live vaccine (21). Priming at one week of age was also considered to avoid vaccination shock and poor intestinal immunity in day old birds (66). Booster dose was given intranasally at three weeks of age targeting the coarse spray administration of cell culture adopted vaccine as reported previously (22). The booster vaccination was done after two weeks of priming because rPICV vaccine needs two to three weeks to provoke best immune response (personal communication with Dr. Ly) Immunogenicity of the codon-optimized bivalent PICV-SKM121 recombinant vaccine was demonstrated by the production of high SN antibody titers. In future *in vivo* experiments, we plan to further characterize the effect of the dose of the recombinant PICV-TARV vaccines on antibody titer and response to reovirus challenge in turkeys as well as to study the efficacy of protection of the PICV-TARV recombinant vaccines against heterologous and homologous challenge viruses.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

(The full-length article will be published in *Pathogens*.)

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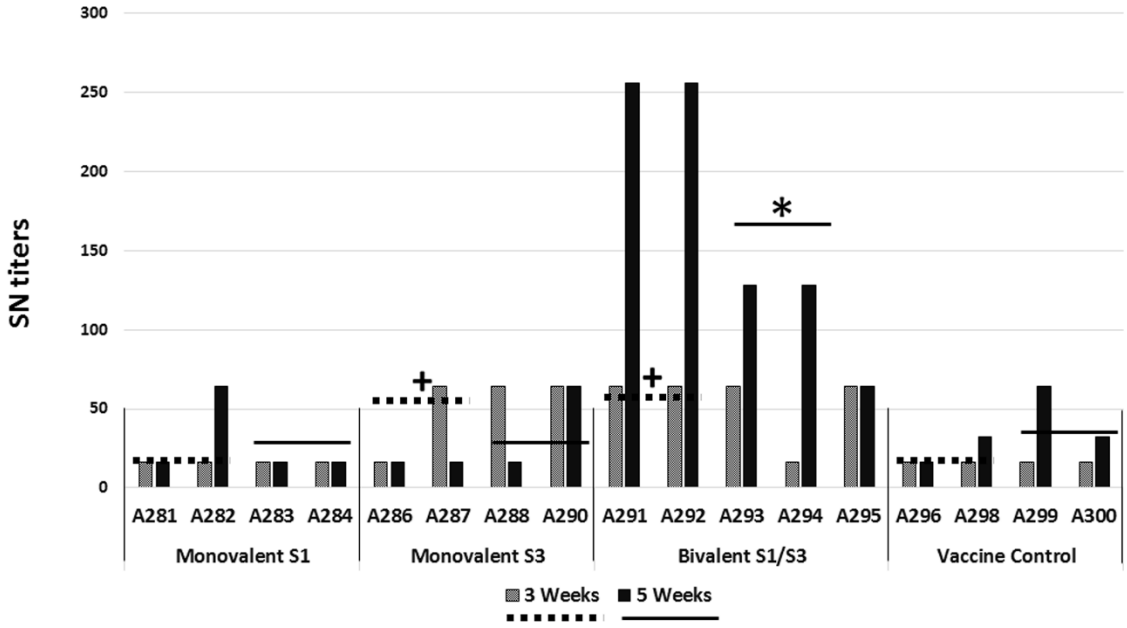
Table 1. Pichinde virus (PICV) recombinant plasmids used to generate vectored viruses.

S. No. of Recombinant vector virus	Insert origin	Recombinant vector virus type	Insert in plasmid 1 pP18S1-GPC/MCS	Insert in plasmid 2 pP18S2-MCS /NP
1	SKM73*	Monovalent	GFP[a]	S1[+] wild type
2		Monovalent	S3[+] wild type	GFP
3		Bivalent	S3 wild type	S1 wild type
4	SKM95*	Monovalent	GFP	S1 wild type
5		Monovalent	S3 wild type	GFP
6		Bivalent	S3 wild type	S1 wild type
7	SKM121*	Monovalent	GFP	S1 wild type
8		Monovalent	S3 wild type	GFP
9		Bivalent	S3 wild type	S1 wild type
10	SKM121*	Monovalent	GFP	S1 codon optimized
11		Monovalent	S3 codon optimized	GFP
12		Bivalent	S3 codon optimized	S1 codon optimized
13		Control	GFP	GFP

[a]GFP= Green Fluorescence Protein.
[+] S1 and S3 are genes inserted in to PICV plasmids.
*SKM73, SKM95 and SKM121 are turkey arthritis reoviruses whose S1 and S3 genes were inserted into PICV plasmids.

Figure **1.** SN antibody titers of individual birds of different groups at different ages at 3-and 5-weeks of age in different groups: MonovalentS1: Monovalent PICV-SKM121 S1 (codon-optimized) recombinant vaccine; MonovalentS3: Monovalent PICV-SKM121 S3 (codon-optimized) recombinant vaccine; Bivalent S1/S3: Bivalent PICV-SKM121 S1/S3 (codon-optimized) recombinant vaccine; Vaccine Control: PICV vaccine with no insert (control). A281 to A300 represent tags of each individual bird. The dotted lines represent the mean values at 3-week-old and the solid lines represent the mean values at 5-week-old. The values of Monovalent S3 and Bivalent S1/S3 groups were significantly higher than the other two groups at 3-week-old (plus sign) and values of Bivalent S1/S3 group were significantly higher than the other three groups at 5-week-old (asterisk sign) using the Mann-Whitney U test at $P<0.05$.



INFECTIOUS LARYNGOTRACHEITIS CASES IN THE FRASER VALLEY OF BRITISH COLUMBIA 2017 TO 2020

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SUMMARY

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

Infectious laryngotracheitis is an acute, highly contagious respiratory disease caused by an avian herpesvirus (Gallid Herpesvirus 1) (2, 3). There is no evidence of vertical transmission, but the virus can be transmitted horizontal from direct bird-to-bird contact (2). The virus can also be spread in fomites through humans, shared equipment, feed and water sources, and vermin (2, 3). Infected birds start to show clinical signs 5 to 12 days after natural exposure (2, 3). Birds that have recovered from an active infection or have been infected subclinically can become life-long carriers (2). Latent virus can be reactivated when the birds are stressed and/or immunosuppressed (2). The disease can persist for up to six weeks in the flock, and losses are associated with poor performance and mortality (3).

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

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

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

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

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

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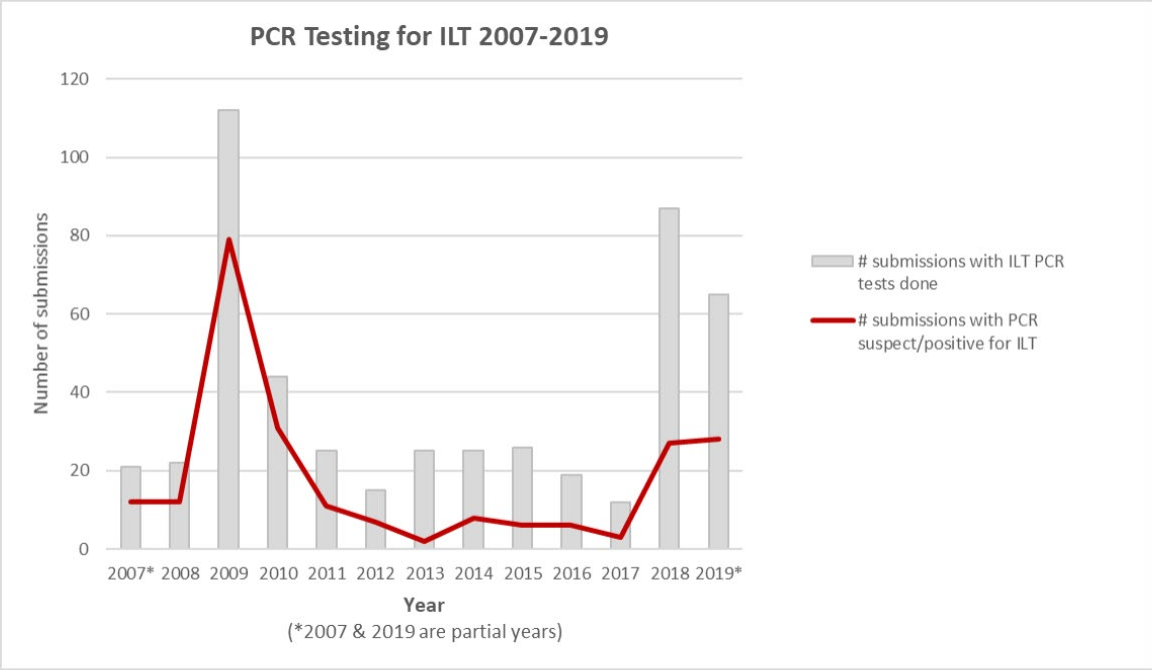
Figure 1. PCR testing and suspect/positive ILT results for submission from 2007-2019 in British Columbia (1).



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

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SUMMARY

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

INTRODUCTION

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

CLINICAL SIGNS

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

GROSS LESIONS

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

DISCUSSION

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

DETECTION AND COMPARATIVE QUANTIFICATION OF *GALLIBACTERIUM ANATIS* AND *MYCOPLASMA SYNOVIAE* STRAINS FROM LAYER FLOCKS USING QPCR METHOD

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SUMMARY

Mycoplasma synoviae is one of the most common preconditions for secondary bacterial infections in poultry. It causes subclinical infections of the upper respiratory tract, which can develop into systemic infections and result in reduced and poor-quality egg production. In such cases, *Gallibacterium anatis* as an opportunistic pathogen often causes clinical infections characterized by depression, diarrhea, respiratory syndrome and decreased egg production. The aim of this study was to determine possible correlation in the prevalence of *G. anatis* and *M. synoviae,* as well as mutual correlation of their quantity. The research was done on sixteen layer flocks from eight different farms. The tracheal swab samples were analyzed using qPCR assay. The results showed statistically significant correlation in the analyzed samples regarding the prevalence of both pathogens on the farms, with significantly higher average levels of *G. anatis* compared to *M. synoviae*. No correlation regarding quantity of each pathogen was detected.

INTRODUCTION

Infections caused by *Gallibacterium anatis (G. anatis)* and *Mycoplasma synoviae (M. synoviae)* can lead to great economic losses in the poultry production. *M. synoviae* is one of the most common preconditions for secondary infections, while *G. anatis* easily spreads from the upper to lower parts of the respiratory system or ascendingly through the oviduct to the ceolomic cavity, and causes severe infections (1).

G. anatis is an opportunistic pathogen that can cause clinical infections characterized by depression, diarrhea, respiratory syndrome and decreased egg production (1,2). However, the infection is frequently subclinical and easily overlooked (3). There are many predisposing factors such as impaired immunity, hormonal status, stress, poor zoohygienic conditions and coinfections, which can lead to systemic infections and high mortality rates (1). The morbidity and mortality rates vary in naturally infected animals, while in experimentally infected immunosuppressed layers the mortality can increase up to 73% (4). Due to inadequacy of the conventional diagnostic methods for detection of *G. anatis*, the pathogen is often difficult to isolate and therefore misdiagnosed. Consequently, a highly specific, sensitive and reproducible qPCR method was developed (2), which was also used in this study in order to identify and quantify the pathogen.

M. synoviae causes subclinical infection of the upper respiratory tract, which can lead to secondary respiratory and systemic infections. It can also cause decreased egg quality and production, as well as lameness and severe arthritis (5). *M. synoviae* strains have tropism for different tissues, but the synergism between arthrotropic and salpingotropic strains and other respiratory pathogens has been documented in several studies (6, 7). As opposed to the respiratory and arthropathic forms, the last 20 years strains with oviduct tropism, which are associated with egg deformities, have been increasingly detected (8). Respiratory form of the disease can be characterized by rales, but is most commonly asymptomatic (5). Morbidity in chicken flocks varies between 2 and 75%, while mortality is usually less than 1% (5), which consequently results in high condemnations, decreased weight gains and feed efficiency. Commercial ELISA assays are often used for routine flock monitoring, while the identification is done using different PCR-based methods (5).

Both bacteria are most commonly located in the upper respiratory system and frequently cause subclinical infections, therefore the aim of this study was to determine their prevalence, as well as mutual correlation of their quantity in the studied laying hen flocks.

MATERIALS AND METHODS

Sampling and DNA isolation. In this study, sixteen flocks from eight laying hen farms were investigated. The age of the flocks varied from 8 to 66 weeks of age (Table 1). Five tracheal swabs per flock were sampled and stored at -20°C until the DNA

isolation. DNA was isolated from every sample individually using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Co., St Louis, MO, USA) according to the manufacturer's instructions. Isolated DNA was stored at -20°C until further analysis.

Quantification of the bacteria was done using GoTaq Probe qPCR Master Mix (Promega, Madison, WI, USA). Used primers and probes were specific for *G. anatis* (*gyrB*: F-5'-CGATTGTGTCCGTTAAAGTGC, R-5'-TGCAAACGCTCACACCAACTG, P-5'-FAM-CTGGTTTCTTCCGAAGTGAAAAGTGTAGTGGA-BHQ1) and *M. synoviae* (16S-23S rDNA ISR: F-5'-CTAAATACAATAGCCCAAGGCAA, R-5'-CCTCCTTTCTTACGGAGTACA, P-5'-FAM-AGCGATACACAACCGCTTTTAGAAT-BHQ1). Quantification protocols were performed as described by Wang et al. (2016), and Raviv and Kleven (2008). The total volume of the reaction mixture was 15 µL. Each sample was analyzed in duplicate, while *G. anatis* and *M. synoviae* samples of known concentration were performed in triplicate in parallel for the purpose of absolute quantification. The analyses were carried out using Mx3005P instrument (Stratagene, USA).

Statistical analysis. The statistical analyses were done in Statistica 13.5.0.17. (TIBCO Software Inc.) software. The normal distribution was tested using Kolmogorov-Smirnov test, and the average number of genome copies was tested using Mann-Whitney U test. Correlation between the pathogen copy number and the correlation between the prevalence of both pathogens on each farm were analyzed using correlation matrices. Statistical significance was set at level $p < 0.05$.

RESULTS

Both pathogens were detected in the majority of the studied flocks, with the exception of Flock 11 (Farm E) and Flock 15 (Farm H) where *M. synoviae* was not detected, and Flock 8 (Farm C) where none of the pathogens was detected, possibly because of the young age of the flock (Table 1). The results showed higher mean copy numbers per sample for *G. anatis,* in most cases (Table 1), and a significantly higher average copy number of *G. anatis* on all farms (data not shown). There was no correlation between the pathogens in regard to the quantity of bacteria per swab (Figure 1a). However, results showed statistically significant correlation ($p = 0.032$) in the prevalence of *G. anatis* and *M. synoviae* (Figure 1b).

DISCUSSION

The objective of this study was to determine the possible correlation in the prevalence of *G. anatis* and *M. synoviae,* and mutual correlation of their quantity in the tracheal swabs of laying hens. Both bacteria colonize the respiratory system, which can result in clinical infections with high production losses. Several studies have shown that coinfections with *M. synoviae* lead to severe clinical symptoms (10-12), although no correlation has been detected between *M. synoviae* and *G. anatis* to date.

Results of this study showed statistically significant correlation ($p = 0.032$) in the prevalence of *G. anatis* and *M. synoviae* (Figure 1b), which confirms the possible link between the studied pathogens. *G. anatis* was detected in almost all flocks and showed higher titers per sample than *M. synoviae,* in most cases (Table 1), which is in favor of the fact that *G. anatis* is a commensal microorganism in the upper respiratory system where it can be found in a high amount without causing a clinical infection. There was no correlation between the quantities of the pathogens in the samples. To our knowledge, there were no clinical manifestations of infections at the time of sampling on any of the studied farms.

Immunoprophylaxis is one of the most important technological measures in the prevention of poultry diseases. Live attenuated *M. synoviae* vaccine showed high efficacy in the prevention of the clinical infections and elimination of the wild strains on farms (13). On the other hand, *G. anatis* strains are characterized by high genetic variability, which complicates the production of a universal commercial vaccine, although different immunogens are being investigated as possible vaccine candidates (1). The results of this study showed a high prevalence of both *G. anatis* and *M. synoviae*, and confirmed the need for regular monitoring on poultry farms. Since *M. synoviae* most frequently causes subclinical infections, the pathogen is often longitudinally spread through the flocks within a farm, which leads to economic losses. Implementation of the adequate methods for early detection and control of the bacterial pathogens on poultry farms can lead to better prevention of the diseases, which will result in healthier flocks and improved production rates. As the prevalence of *Gallibacterium* is influenced by the production system and the level of biosecurity measures on farms (14), more detailed research considering the production management should be conducted.

ACKNOWLEDGEMENTS

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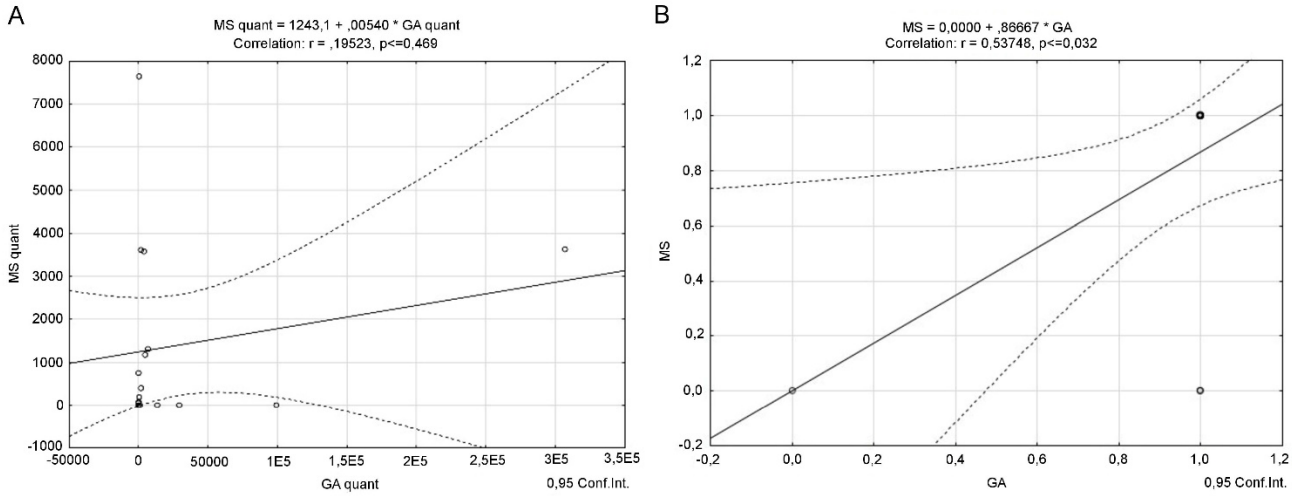
Table 1. Mean values of *G. anatis* (GA) i *M. synoviae* (MS) genome copies per swab (mean ± SD) with age f the studied flocks.

Farm	Flock	GA	MS	Age (weeks)
A	1	388 ± 534	7645 ± 5166	41
	2	307040 ± 683548	3628 ± 2441	41
	3	13596 ± 25438	3 ± 3	49
	4	29374 ± 63502	1 ± 3	49
	5	4856 ± 8633	1175 ± 1114	60
B	6	111 ± 158	73 ± 127	60
	7	502 ± 640	191 ± 177	19
C	8	0 ± 0	0 ± 0	8
	9	69 ± 71	754 ± 893	30
D	10	41 ± 65	51 ± 98	66
E	11	1126 ± 1838	0 ± 0	33
F	12	1804 ± 2219	400 ± 363	29
	13	1800 ± 3266	3615 ± 2991	36
G	14	3974 ± 5222	3578 ± 3914	38
H	15	99196 ± 197976	0 ± 0	26
	16	6863 ± 8341	1313 ± 2599	42

Figure 1. A Correlation between the *G. anatis* (GA) and *M. synoviae* (MS) number of genome copies per swab on each farm. B Correlation of GA and MS prevalence on each farm.



ANALYSIS OF INFECTIOUS BRONCHITIS VIRUS IN CALIFORNIA BACKYARD CHICKENS

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SUMMARY

Infectious bronchitis virus (IBV) contributes to respiratory, reproductive and renal disease associated production losses and in some cases mortality in chickens, overall leading to significant losses in the poultry industry. California harbors significant numbers of commercial poultry farms as well as numerous small flocks, often closely situated, leading to transmission and exchange of infectious agents via spontaneous and vaccine-associated infections between the two. Here we examined all submissions of backyard chickens to the Davis (Northern California; NorCal) and San Bernardino (Southern California; SoCal) branches of the California Animal Health and Food Safety laboratory system during the first three months of 2019. All cases were analyzed for a) prevalence of IBV in the trachea, kidney and cecal tonsils by PCR and immunohistochemistry, b) histologic lesions in the trachea and kidney, c) sequence data of IBV PCR positive cases, d) geographic locations of positive flocks, and e) contribution of IBV to disease and mortality of backyard chickens. Trachea, kidney, and cecal tonsils were collected for qRT-PCR, histology, immunohistochemistry (IHC) and sequence analysis. A total of 50 chickens out of 123 tested positive for IBV by qRT-PCR. The cecal tonsil was the most frequently positive tissue by qRT-PCR and IHC. Lymphoplasmacytic tracheitis was the most frequent histopathologic finding in 24 of 39 birds. Infectious bronchitis virus played a primary role or as a synergistic effect in the mortality of chickens succumbed by other infectious diseases. The sequences of IBV detected in 22 birds were analyzed and 14 strains were most similar to CA1737. One strain each matched Conn46, Cal99, and ArkDPI, and the remaining five did not have a substantial match to any available reference strains.

INTRODUCTION

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

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

This study plots the prevalence of IBV in backyard chickens submitted to the Davis (Northern California; NorCal) and San Bernardino (Southern California; SoCal) branches branch of the California Animal Health and Food Safety Laboratory System, UC Davis, for necropsies and provides information on the pathological and diagnostic features of IBV, the relation of IBV to causes of mortality, and circulating strains in the field.

MATERIALS AND METHODS

IBV sampling. Two sets of trachea, kidney and cecal tonsil samples were collected during the necropsy of every BYC submitted to the NorCal and SoCal laboratories for diagnostic investigation between January 1st and March 31st of 2019. One set was fixed in 10% buffered formalin for histology and IHC. The other set of tissues were stored at -80°C for molecular analysis.

qRT-PCR. The frozen trachea, kidney, and cecal tonsil tissue pool was used for the initial IBV screening in each case and when positive, the tissues

were tested separately. Total RNA was extracted using a commercial magnetic beads kit (Ambion 1836, Thermo Fisher, Waltham, U.S.) following the recommendation of the manufacturer for automatic extraction using a Biosprint 96 well machine (Qiagen, Germantown, U.S.). RNA was subjected to qRT-PCR assay (Thermocycler, ABI 7500 fast, Thermo Fisher, Waltham, MA, U.S.) targeting a 143bp fragment of the 3' non-coding region of the viral genome (6). A Ct value of 35 was used as the cut-off point; any value <35 was considered "positive" (+), and 35-40 as "indeterminate" (I; inconclusive). A bird was considered 'positive' (+) when at least one of the IBV PCRs (pool, trachea, kidney or cecal tonsils) was positive (Ct<35).

Standard PCR and phylogenetic analysis. Kidneys and/or cecal tonsils with qRT-PCR Ct values of <32 were subjected to RT-PCR targeting a 768bp fragment of the S1 gene (7). For PCR amplification, a commercial One-Step RT PCR kit (Qiagen, Germantown, U.S.) was used following the manufacturer's instructions. The resulting amplicons were visualized on a 4% agarose gel, purified using Amicon Ultra (Millipore Sigma, Temecula, U.S.) 0.5 mL centrifugal DNA filters, and sequenced by the Sanger method. Forward and reverse sequences for each sample were aligned using the program Geneious Prime (version 2020.0.5, Biomatters Inc, San Diego, U.S). The resulting contiguous sequences were submitted to GenBank and assigned the following accession numbers: MT427357 – MT427389. A multiple sequence alignment of the IBV S1 gene fragment was produced in Geneious Prime using the MUSCLE alignment algorithm. The following reference sequences and associated Genbank accession numbers were included in the alignments: CA1737 (DQ912830), Beaudette (AJ311317), Mass41 (FJ904713), Conn46 (FJ904719), Delmarva 1639 (MK878536), Cal 557 (FJ904715), Iowa 97 (GU393337), Cal99 (AY514485), Ark99 (MH779860), ArkDPI101 (EU418975), ArkDPI (GQ504720), CA1737 (DQ912830) and Del072 (GU393332). Phylogenetic analysis using a Maximum Likelihood method was performed with the program MEGA 6 (8). The Tamura 3-parameter model of nucleotide substitution with a discrete Gamma distribution was used. Bootstrap values were calculated using 1000 pseudo-replicates.

Histopathology was performed on HE sections of trachea and kidneys in every bird with available tissues. The scoring of histological lesions were based on mild (+1), moderate (+2) and severe (+3) lesions based the presence of a) single-cell necrosis in the epithelium, and b) leukocytic infiltrations in the tracheal epithelium and lamina propria in the trachea. The criteria in the kidney were a) tubular necrosis characterized by distention of convoluted tubules, degeneration/coagulative necrosis of the tubules, and sloughed epithelial cells and heterophils in the lumen, b) lymphoplasmacytic interstitial infiltrations, and c) gout.

Immunohistochemistry for IBV was performed using monoclonal antibodies described previously (9). Procedures included 3% hydrogen peroxide treatment in water for 10 min after deparaffinization and rehydration of the tissue sections then heating the slides in a decloaker for 10 min, at 121°C for antigen unmasking in Diva Decloaker Solution (Biocare Medical, DV2004G1). For primary antibody, a cocktail of each monoclonal (9.19 and 9.4) was diluted 1:10,000 each in DaVinci Green antibody diluent (Biocare Medical, PD900M), and incubated for 45 min at room temperature (RT). Envision + Anti-Mouse HRP-Polymer (Dako, K4001) was applied to slides as secondary antibody and incubated for 30 min at RT. TBS-Tween was used for rinses between steps. Slides were incubated for 15 min at RT for visualization by AEC chromogen (Dako Ready-to-Use, K3464), counterstained by Mayer's Hematoxylin, blued, and rinsed in deionized water before applying aqueous mounting medium and finally permanent coverslips.

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

Case data. The geographic locations of the IBV-positive chicken flocks were mapped at the county level using a web service mapping tool GeoNames® (10). Information on breed, sex, age, cause of death (natural/sudden death or euthanasia), and the diagnosed primary condition were recorded for the IBV-positive chickens.

RESULTS

qRT-PCR. Of the tested 123 chickens from NorCal and 46 chickens from SoCal, a total of 50 chickens tested positive: 20/123 (16%) from NorCal and 30/46 (65%) birds from the SoCal laboratory. Seven chickens (one from NorCal and six from SoCal) had an indeterminate value (I = Ct>35) in the pool and/or any singly tested tissue and were considered negative. Of the individually tested trachea, kidney, and cecal tonsil, the latter was the most frequently positive with 49 of the 50 tested cecal tonsils being positive. The kidney was the second with 19 out of 49 tested, and lastly 8 out of 49 tested tracheas were

positive. The one chicken that tested negative on the cecal tonsil tissue was also negative in the trachea and kidney tissues separately although the initial pooled sample was PCR positive. This bird was also positive for IBV in the kidney by IHC.

Standard PCR and phylogenetic analysis. Sequencing was performed on 27 tissues (7 kidneys, 20 cecal tonsils with Ct<32) from 22 birds. Phylogenetic analysis revealed 14 genotypes with 91% - 96% homology to CA1737 (Figure 1). Since most of these isolates are below 95% homology to CA1737, they can be considered variants. One bird from SoCal (15-SanB) had an IBV strain with 99.9% similarity to Conn 46 strain. The isolates of 8-Davis and 19-Davis were most similar to Cal99 and ArkDPI strain with 89% and 88% of identity, respectively. Five birds (4-Davis, 5-Davis, 9-Davis, 13-Davis, and 16-Davis) had strains that did not have substantial matches to any of the reference strains. Those strains showed the closest relationship to Cal557 with 81% - 84% homology.

Histopathology. The most frequent histological lesion in the trachea was lymphoplasmacytic tracheitis observed in 24 of 39 available tracheas and the inflammation grade was mild "+1" (n=12), moderate "+2" (n=5) and marked "+3" (n=7). Epithelial necrosis was seen in only two birds and they were graded as "+1" and "+3.".

In the kidneys, lymphoplasmacytic interstitial nephritis was the most frequent diagnosis in 12 of 43 available kidneys, of which nine were scored "+1" and three were "+2." Mild (+1) tubular necrosis (Figure 4) was observed in eight and gout in five of the 43 examined kidneys. Tubular dilation was observed in only one kidney and was graded as "+1."

Immunohistochemistry. Eight of the 47 (17%) examined tracheas were immunopositive and the labeling intensity was "+1" in six and "+2" in two chickens. The cells with specific immunoreactivity were primarily the submucosal macrophages. Twenty-two of 47 (47%) examined kidneys were positive for IBV and the immunolabeling intensity was "+1" in 10 birds, and "+2" in nine and "+3" in three chickens in the cytoplasm of convoluted tubular epithelial cells and scattered macrophages. The cecal tonsil was the organ with the most frequent positive cases including 24 of 44 (55%) available cecal tonsils and the intensity was classified as "+1" in 16, "+2" in seven, and "+3" in only one bird. Enterocytes and a few macrophages in the lymphoid follicles were immunolabeled by IHC in the +3 bird.

Case data. The geographic distribution of the IBV PCR positive chickens within the state of California is shown in Figure 2. The cases were sparsely distributed in 12 and 6 counties from NorCal and SoCal, respectively. Santa Clara (NorCal) and San Bernardino (SoCal) were the counties with the most positive cases with four and eight birds from separate flocks, respectively.

Of the 50 chickens the majority were females (n=42), five were males and three chickens were not specified. Majority of the chickens (24) were ≥1 year of age, 14 chickens were < 1 year-of-age and the age of 12 chickens were not available. Causes of death were sudden death in 23 chickens and euthanasia in 11, while 16 were not recorded. Lymphoproliferative disease (LPD) was the main diagnosis in 20 of the 50 IBV positive birds in both laboratories based on the histologic infiltration of pleomorphic lymphocytes in the visceral tissues, peripheral nerves, and/or brain. Ten chickens had neoplastic disease including carcinomatosis of ovarian origin (n=6), sarcoma (n=2), brain tumor (presumptive astrocytoma) (n=1), and oral squamous cell carcinoma (n=1). Respiratory tract disease was the primary cause of mortality in eight chickens which mostly had variable degrees of tracheitis. Renal disease was diagnosed as the primary disease in three cases and all had tubular necrosis and gout. Other infectious diseases as primary diagnoses are septicemia (n=3), bacterial salpingitis and peritonitis (n=1), coccidial typhlitis (n=1), and anemia associated with severe Northern fowl mite infestation (n=1). Non-infectious diseases were nutritional myopathy, hemorrhagic liver disease, coelomic hemorrhage, and trauma observed in one bird each.

CONCLUSIONS

This article represents the first long-scale study to characterize IBV infection in BYF. The results suggest that IBV is widely distributed in backyard chickens in Northern and Southern California, the virus persists in the cecal tonsil, CA1737 is the most prevalent genotype similar to the commercial poultry in the state, and IBV may cause direct effect in the trachea and kidney or be a synergistic factor in the death of backyard chickens. Furthermore, the findings emphasize that small flocks can serve as an environment where IBV can mutate and evolve into new, likely more pathogenic variants.

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

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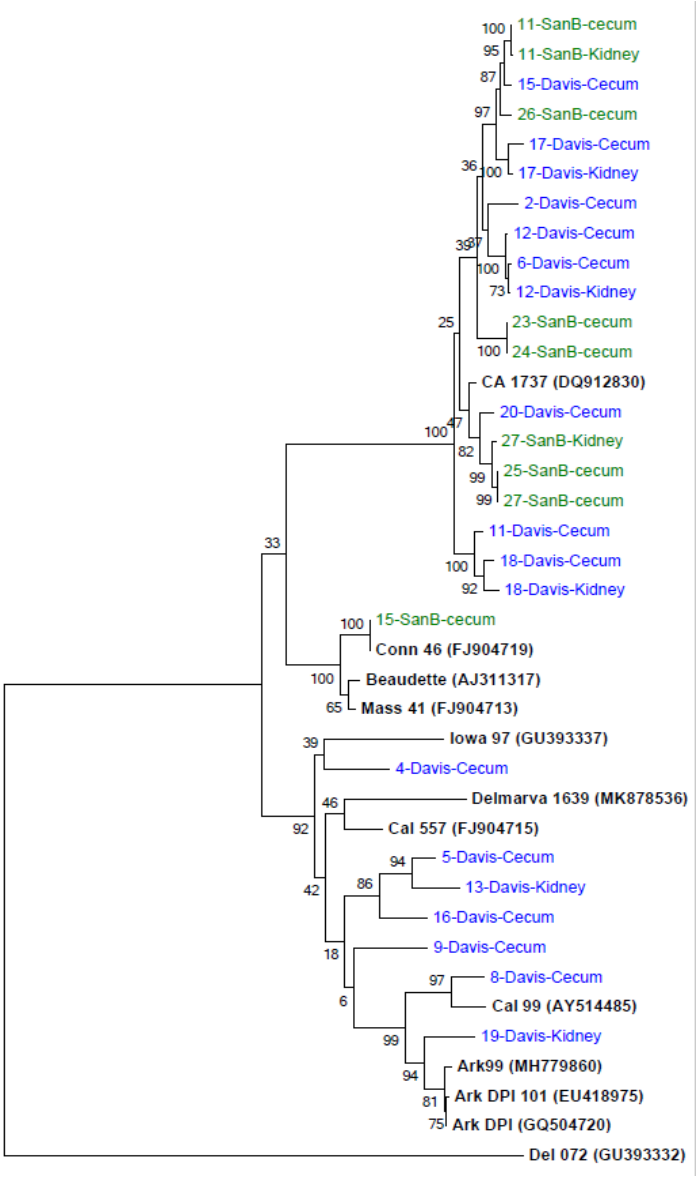
Figure 1.


11-SanB-cecum
11-SanB-Kidney
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12-Davis-Cecum
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23-SanB-cecum
24-SanB-cecum
CA 1737 (DQ912830)
20-Davis-Cecum
27-SanB-Kidney
25-SanB-cecum
27-SanB-cecum
11-Davis-Cecum
18-Davis-Cecum
18-Davis-Kidney
15-SanB-cecum
Conn 46 (FJ904719)
Beaudette (AJ311317)
Mass 41 (FJ904713)
Iowa 97 (GU393337)
4-Davis-Cecum
Delmarva 1639 (MK878536)
Cal 557 (FJ904715)
5-Davis-Cecum
13-Davis-Kidney
16-Davis-Cecum
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Cal 99 (AY514485)
19-Davis-Kidney
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Ark DPI 101 (EU418975)
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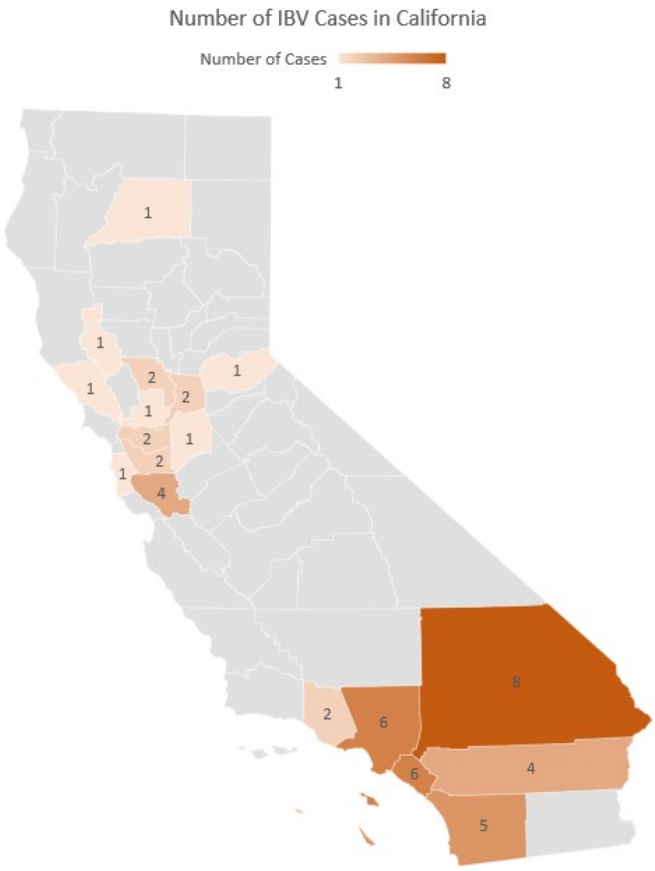
Figure 2.


Number of IBV Cases in California
Number of Cases
1
8
1
1
2
2
1
1
2
1
2
1
4
8
2
6
6
4
5

FOWL AVIADENOVIRUS C-4 IN A CALIFORNIA SMALL FLOCK

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SUMMARY

FAdV-4 was detected in a remote backyard flock in California where seven from a mixed flock of 30 chickens varying in breeds and ages (six months to two years old) were found dead within a week without premonitory signs. One additional bird died after the flock was relocated to fresh pasture, bringing the total mortality to 8/30 (27%). On postmortem examination of three birds, the only significant lesion was subtle petechiations throughout the liver of one bird, while the other two had diffusely dark mahogany livers. Histopathology of two chickens revealed typical adenoviral acute hepatic necrosis with many hepatocytes containing large, mostly basophilic, smudgy intranuclear inclusions. Negative staining and transmission electron microscopy identified 90 nm diameter adenovirus particles. Virus isolation and genomic sequencing revealed strains with 99.9% homology to FAdV-4 isolates reported from China. This reports the emergence of C-4 in the United States and in a backyard chicken flock. Furthermore, the chickens affected here were all adults and exhibited a variation of serotype 4 in which IBH was present but not hydropericardium.

INTRODUCTION

Fowl aviadenoviruses (FAdVs) cause a triad of diseases in chickens: inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), and adenoviral gizzard erosion (AGE) (1). Of these, IBH is the best described given the frequent and globally widespread outbreaks, followed by HHS. FAdV species D and E are the most commonly detected species in IBH outbreaks. Serotype 4 of FAdV species C is associated with HHS. Both IBH and HHS primarily affect young chickens. Among commercial poultry, outbreaks in broiler chickens are the most common, followed by layers and broiler breeders. Reduced performance and reproduction are the primary cause for economic losses, although mortality can reach 30% with IBH and higher with HHS (1).

FAdV-4 strains have caused epidemics and severe losses in China since 2015, as a result of HHS outbreaks in commercial flocks (2). To our knowledge, FAdV-4 associated outbreaks have not been reported in the U.S. commercial poultry industry. Furthermore, studies and surveillance conducted over the past 10 years in California, and over the past five years in seven additional states throughout the U.S., have not reported IBH or HHS in backyard chickens (3, 4). The tremendous increase in small backyard poultry flocks throughout the U.S. has introduced an unpredictable interface with commercial facilities, and we are constantly monitoring the disease dynamics between these two entities.

MATERIALS AND METHODS

In April of 2020, increased mortality was reported in a backyard flock of 30 hens. The flock consisted of multiple breeds of laying hens from six months to two years old housed on pasture in a mobile coop with access to outdoors. Seven sudden deaths occurred within a week. The chicken coop was then cleaned and relocated to adjacent fresh pasture. An eighth chicken died within several days of relocation, then no further mortalities were reported. Three carcasses from the first deaths were collected at random for testing: gross pathology was recorded on all three chickens and ancillary testing including routine histology and PCR was performed on liver from two chickens.

Electron microscopy was performed on liver samples from two chickens. Liver homogenates were prepared from both chickens and examined under contrasted phosphotungstic acid (negative staining). Transmission microscopy on plastic embedded tissues was also performed on livers of both birds.

For liver real time PCR (qPCR), a 143-bp region of the *hexon* gene using primers published previously (5) and a newly designed probe (FAM-CAGATGWCTGACGCSGASTAC-BHQ1) was amplified. Virus isolation was performed on one chicken. The liver suspension was inoculated on chicken embryo liver cell culture. CPE was observed after 5 d of incubation at 37°C. Following ultracentrifugation, total nucleic acid was extracted (MagMax pathogen RNA/DNA kit; Thermo Fisher). A DNA library was constructed (Ligation sequencing SQK-LSK109 kit; Oxford Nanopore Technologies [ONT]). The library was loaded onto a Flongle flow cell (ONT) and run on a sequencer (MinION; ONT)

using default parameters for 24 h. Base-called FASTQ files containing "pass" reads (Q-score ≥7) were loaded into Geneious Prime (v.2020.0.5).

RESULTS

On postmortem, all three chickens were in good body condition with abundant adipose and active ova with a shelled egg in the uterus. Gross lesions in all were darkened organs, pulmonary edema, and one chicken had generalized subtle petechiation in the liver (Fig. 1 inset). In the other two chickens the kidneys were swollen and had subtle, pale, tubular highlights. There were minimal crop and gastric feed contents. The koilin layer of the ventriculus peeled easily and was partially detached. One hen had mild egg yolk peritonitis. Brain, peripheral nerves, heart, lung, trachea, liver, kidney, spleen, ova, adrenal gland, skeletal muscle, pancreas, and gastrointestinal tract tissues from two chickens were collected, processed routinely for histology, and stained with hematoxylin and eosin.

On histology, the livers of both birds had severe acute necrosis with hemorrhages and fibrin exudation (Fig. 1). Hepatocellular nuclei were frequently obliterated by large basophilic INIBs or occasionally by eosinophilic INIBs and peripheralized chromatin (Fig. 1). Pulmonary capillaries had fibrin thrombi. The koilin was lifted off, had segmental degeneration, and there was mild heterophilic (1/2) or lymphocytic (1/2) ventriculitis. The kidneys in both birds had moderate edema, tubular degeneration with proteinaceous luminal material, and urate stasis. In the spleen, there was mild-to-moderate lymphoid depletion, fibrin exudation, and histiocytosis.

Negative staining detected non-enveloped, icosahedral, hexagonal, 82.2 (SD 5.5) nm in diameter virus particles in both livers. Non-vertex capsomeres measured ~1-.0 (SD 1.3) nm. Double-vertex fibers were observed infrequently. Density of adenoviral particles in one of the specimens was calculated to be 7,780 per μm^2. On examining liver tissue embedded in plastic, hepatocellular intranuclear virus replication and assembly complexes were composed of electron-dense viroplasm and maturating and mature virions displaying loose-to-compact aggregation designated as paracrystalline arrays. Denatured nuclear chromatin formed asymmetric electron-dense amorphous aggregates (Figs. 2, 3).

Liver tissue qPCR confirmed fowl aviadenovirus 4 in both samples. Virus isolation and sequencing data led to the generation of a consensus sequence corresponding to the entire FAdV-4 genome (43,717 bp) at a coverage of 200X. On BLASTN, the sequence identified 99.99% homology with GenBank accession MG547384, corresponding to a C-4 isolate from China (Fig. 4). Our isolate was submitted to GenBank (accession MT813039).

DISCUSSION

There are 12 known serotypes of FAdVs, belonging to five distinct species. Serotypes isolated from field outbreaks in the poultry industry throughout the world show a predominance of serotype 8 in IBH cases and serotype 4 in HHS cases (1). Although the hepatic lesions are equivalent in IBH and HHS, the distinguishing factor between these two conditions is the presence of hydropericardium and cardiac lesions described with HHS. In our FAdV C-4 associated deaths in a flock of adult backyard hens, none of the three examined chickens had classical gross lesions described in commercial birds with adenoviral hepatitis, with the exception of the petechiation in the liver of one chicken. The outbreak described here of C-4 in a backyard flock of adult hens with classical IBH lesions, but not HHS, and with mortality close to what is expected (30%) in HHS outbreaks, adds to the complexity of categorizing FAdV-associated diseases (6).

The FAdV C-4 strain isolated here lies in a cluster of Chinese strains that are 99.99% identical, differing in only 6 bp in the genome, and with no amino acid changes: F-Vac, JSJ13, NIVD2, HLJFAd15, and ZJ2015 (7). Of these, F-Vac is the strain isolated from a contaminated Newcastle disease viral vaccine; the remainder are wild strains isolated from the outbreaks in China (7). The role of contaminated attenuated live Newcastle disease viral vaccines has been documented, and these vaccines are the suggested cause of the rapid and geographically distinct spread of FAdV outbreaks in China (8). The spread of the outbreaks was exacerbated mostly by contamination by both FAdV and CAV (8). The suggested vaccine associated with these outbreaks is in use in the U.S., although we are not currently aware of any contamination issues. The spread of FAdV through contaminated vaccines does not seem likely to be a primary route of exposure for backyard chickens, given that small hobby flocks tend to have minimal vaccination strategies. If at all, backyard birds are vaccinated against Marek disease, but rarely against other diseases such as Newcastle disease or adenoviral infections. California commercial poultry operations, may however be at risk.

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Figures 1-3.

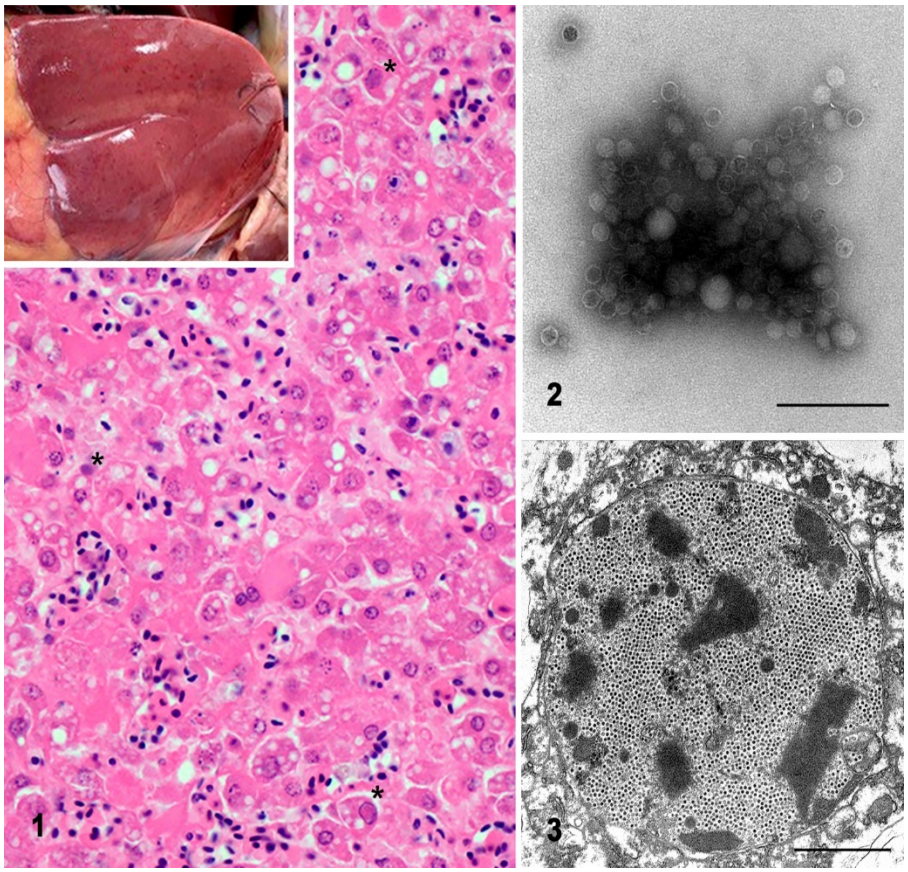
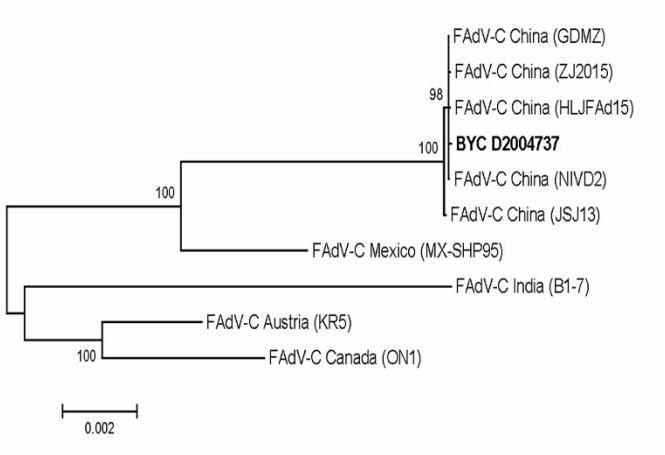


Figure 4.


FAdV-C China (GDMZ)
FAdV-C China (ZJ2015)
98
FAdV-C China (HLJFAd15)
100
BYC D2004737
FAdV-C China (NIVD2)
100
FAdV-C China (JSJ13)
FAdV-C Mexico (MX-SHP95)
FAdV-C India (B1-7)
FAdV-C Austria (KR5)
100
FAdV-C Canada (ON1)
0.002

ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF A BIOCHEMICALLY ATYPICAL *SALMONELLA* TYPHIMURIUM IN US POULTRY

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SUMMARY

Surveys of *Salmonella enterica* on some US poultry farms have recovered serotype Typhimurium with an atypical biochemical profile. These isolates are arginine dihydrolase, hydrogen sulfide, sorbitol, and melibiose negative and do not identify as *Salmonella* spp. on API® 20E identification kits (bioMerieux, France). Serotyping was confirmed by intergenic sequence ribotyping. These isolates were further characterized with typical *Salmonella* Typhimurium strains by whole genome sequencing, multi-locus sequence typing, antibiotic resistance genes, virulence genes and pangenomic analysis. Other than phenotypic differences, the absence of the cyclic AMP regulatory protein gene was the distinguishing characteristic of atypical *Salmonella* Typhimurium.

INTRODUCTION

Annually there are just over 1 million cases of Salmonellosis with over 19,000 hospitalizations causing over 300 deaths per year in the United States (1) accounting for around 11%, 35% and 28% of all foodborne cases, hospitalizations and deaths respectively. The economic burden is estimated at greater than $3.7 billion annually (2). *Salmonella* Typhimurium is the third and the monophasic strain is the fifth most prevalent serotype causing human infection (3).

Classical bacterial identification is performed using a panel of individual biochemical and enzymatic reactions the results of which indicate to the trained microbiologist the genus and species of the organism in question (4). These panels are available in kit form such as API® and Vitek® systems that return a bionumber as a result of the individual tests on the panel (5, 6). This bionumber is then input into a library database and will result in a bacterial identification. Once the identity is confirmed to the species level, serotyping is performed using slide or tube agglutination assays and the White-Kauffmann-Le Minor scheme to identify the serotype (7). With this pipeline it is important to identify the organism as *Salmonella* spp. prior to serotyping to reduce false positives due to cross reactions amongst other bacteria of the family Enterobacteriaceae. Further distinction among isolates of the same serotype can be made using phage typing (8). This technique was utilized in determining host adaptation in *Salmonella* Typhimurium (9) and has proven to be a valuable epidemiological tool (10).

The development of molecular assays including whole genome sequencing (WGS) has increased the methods by which we identify and characterize *Salmonella* strains. Polymerase chain reaction (PCR) assays have been developed that can replace classical methods for identification (11). *Salmonella*, *inv*A PCR was comparable with API for accurately identifying *Salmonella* spp. (12). In our laboratory the Kauffmann-White-Le Minor serotyping scheme has been replaced by Intergenic Sequence Ribotyping (ISR) (13). Serotyping by ISR utilizes sequencing a specific PCR target of *Salmonella* and can distinguish 133 serotypes and 242 unique ISR sequences. Pulse-field gel electrophoresis was the staple of epidemiological analysis prior to WGS and was the basis for the pulsenet system utilized by various agencies responsible for tracking *Salmonella* outbreaks (14). For this process, extracted DNA is cleaved using restriction endonucleases into various lengths and the resulting DNA banding pattern is unique to that strain (15). Multi-locus sequence typing (MLST) has been used in evolutionary analysis of *Salmonella* and epidemiological assessments before and after outbreaks (16, 17). The MLST is determined by sequencing and concatenation of seven housekeeping genes within the *Salmonella enterica* genome. Each housekeeping gene is assigned an allele number based upon the gene sequence. The sequence type of an isolate is determined by the combination of the allelic profile and is assigned a specific MLST number (18).

In today's high throughput and technologically advanced diagnostic laboratories, the molecular methods mentioned previously have replaced traditional biochemical testing. In our laboratory, we

use classical methods of biochemical profiles as a method of phenotyping, in combination with molecular methods for isolate characterization and vaccine candidate selection. As a result of this process, we have isolated a number of biochemically atypical *Salmonella* Typhimurium. These isolates are arginine dihydrolase, hydrogen sulfide, sorbitol, and melibiose negative and do not identify as *Salmonella* spp. on API® 20E identification kits (bioMerieux, France). The Typhimurium serotype was confirmed by ISR and further characterized using whole genome sequencing to assess antibiotic resistance and virulence gene patterns compared to typical *Salmonella* Typhimurium strains. Those laboratories relying solely on classical bacteriological methods may mis-identify *Salmonella* strains (19).

MATERIALS AND METHODS

Bacterial culture and identification. Eighteen *Salmonella* Typhimurium isolates from chick pads or carcass rinses are characterized in this report. They were isolated from broiler chicken complexes covering four companies over five states in the US. Chick pad samples containing feces were swabbed with sterile moist sponges in pools of 3-5 pads per flock and incubated in 150 mL tetrathionate-brilliant green enrichment broth (Becton-Dickinson). Carcass rinse samples were similarly enriched at a 1:10 v/v ratio. Enrichment broth cultures were incubated for 24 ± 2 hours at 37 ± 2 °C. After incubation, enriched cultures are struck to XLT4, MacConkey, and Brilliant Green Agar plates (Remel, Lenexa, KS) for isolating colonies. Suspect *Salmonella* colonies were sub-cultured to Sheep Blood Agar plates (Remel, Lenexa, KS) prior to ISR and biochemical phenotyping. Serotyping by ISR was performed as described by Guard et. al. (13). The raw ISR sequence data was processed using Geneious Prime® version 2019.2.3 and blast searched against a current ISR database of serotype sequences. Biochemical phenotyping was performed using API 20E identification kits (bioMerieux, France) according to the manufacturer's instruction.

Whole genome sequencing. The samples were extracted using the MagMax Core Nucleic Acid Purification Kit (Life Technologies, A32702) and were run through the Illumina® DNA Prep, (M) Tagmentation (Illumina, 20018704) followed by sequencing on the Illumina MiniSeq (Illumina, SY-420-1001). Raw Illumina reads were normalized , trimmed and paired with in-house python script using Snakemake(20), and BBnorm(21), and Trimmomatic(22). Denovo assembly was done on samples using in-house python script and Unicycler(23). Sample assemblies were filtered for only *Salmonella* contiguous sequences using in-house python script and Kraken2 (24). Samples were screened for MLST profiles using in-house python script and MLST (25), followed by screening of resistance and virulence genes with in-house python script and Abricate (26). Pangenome analysis was done by first annotating genes in samples with in-house python script and Prokka (27), followed by in-house python script using pangenome pipeline Roary (28). Core genome maximum likelihood tree was constructed with Fasttree (29). Phylogenetic tree visualization was done in R (30), using packages ggtree, treeio, ggplot2, dplyr and tidytree. Logistic principle component analysis (PCA) done in R with packages logisticPCA, ggplot2, dplyr, readr and ggrepel.

RESULTS

Bacterial culture and identification. The results of biochemical phenotyping are shown in Table 1. Atypical isolates are defined as those not returning an acceptable *Salmonella* spp. identification on the API testing. These atypical isolates are arginine dihydrolase, hydrogen sulfide, sorbitol, and melibiose negative. Also shown with this data set are the sequence types of the MLST and the ISR sequence number. Most isolates are sequence type 19 (16/18, 89%). Two other sequence types are 413 and 2379 (1/18, 5.6% respectively). All atypical phenotypes are ISR sequence 147 (11/11, 100%) while only one (1/7, 14%) typical phenotype is ISR sequence 147. Other typical ISR sequences are 6 (3/7, 43%), 37 (1/7, 14%) and 114 (1/7, 14%). While it appears that ISR sequence 147 correlates to atypical phenotypes, further research into our sequence database has discovered typical phenotypes with ISR sequence 147 (data not shown). Further investigation of this would be needed for statistical relevance.

Whole genome sequencing. The presence or absence of resistance genes of the comprehensive antibiotic resistance database (CARD), show an interesting characteristic amongst atypical isolates. The CARD heat map of Table 2, indicates the absence of the cyclic AMP receptor protein (CRP) within the genomes of atypical phenotypes. No variation is seen in the resistance gene patterns among the atypical phenotypes. The only variation seen with typical phenotypes correlates to the different MLST and differ only in the present of a *ram*A or *tet*B gene. The heat maps of virulence factors shown in Table 3 indicate only major variations in presence/absence virulence patterns along the different MLST identifications. For the pangenome analysis, all genes within this set of isolates were assessed and divided into core and accessory genes (soft core, shell and cloud) groups

(31). Core genes are those present in greater than 99% of all genomes sampled and are usually required for function. These genes are present in all genomes of a given clade. Soft core genes are present in 95% - 98% of all genomes sampled. Shell genes are present in 15% - 94%, and cloud genes are present in less than 15% of all genomes sampled. For this purpose, soft core, shell and cloud genes are grouped as accessory genomes and are acquired genes but may not be necessary for function (32). There were a total of 5752 genes in the Typhimurium genomes of which, 3901 were core genes, 0 soft core genes, 703 shell genes and 1148 cloud genes. The phylogenetic tree of core genomes is shown in Figure 1. The core genes of all *Salmonella* Typhimurium in this study, regardless of phenotype, are clonal except that of sequence type 413. Pangenomic separation of phenotypes is exampled in Figure 2 of the phylogenetic tree for all accessory genomes. Atypical phenotypes remain clonal but are distinguished among typical phenotypes. Principal component analysis (PCA) was used to produce the two dimensional chart of Figures 3a and 3b. From the total 5752 pangenome genes, only 529 were used in the PCA. These 529 genes are well defined cloud genes (not hypothetical) and present in any one isolate. This diagram shows the clonality of the tight cluster of atypical phenotypes with more distantly related typical phenotypes. One interesting note is one typical phenotype (LS20-4075-30) clustering with the atypical phenotypes. This tight cluster in the PCA consists of all ISR sequence 147 isolates. As stated prior, further investigation of ISR sequence 147 typical and atypical strains is needed.

DISCUSSION AND CONCLUSION

The present study has shown that traditional biochemical phenotyping still serves as a valuable tool in characterizing *Salmonella* strains. Contrary to that, it exemplifies the need for molecular identification methods to identify atypical strains that could lead to misidentified pathogens if only traditional methodology is utilized.

The absence of CRP genes within the atypical phenotype is noteworthy. The CRP gene functions to regulate a number of other genes in *Escherichia coli* models (33). One of the main functions is to regulate the metabolism of carbohydrates other than glucose (34). This is a possible explanation for the unusual sugar fermentation reactions seen with the atypical phenotypes of Table 1(35). This gene also has an important role in antibiotic resistance by modulating multidrug efflux pumps in *Escherichia coli* (36). Salmonella virulence is affected by CRP as it regulates Salmonella Pathogenicity Island 1 type-3 secretion system proteins. *Salmonella* Typhimurium strains that lack CRP are non-virulent in a mouse model (37) and avirulent strains of *Salmonella* Choleraesuis deficient in CRP have been used as candidates for live vaccines in swine (38).

We have used pangenomic analysis to characterize and differentiate multiple pathogens in our laboratory. The clonality of the atypical phenotype across core and accessory genes is interesting as these isolates come from multiple complexes in multiple states. Ongoing surveillance will lend to the occurrence of these atypical phenotypes in poultry complexes. Is their presence significant? Would they function in competitive exclusion removing pathogenic serotypes? We are continuing to analyze these isolates by attempting to circularize and complete the sequences.

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Table 1. Biochemical profiles of *Salmonella* Typhimurium and the API® identification.

Isolate	MLST (ST)	ISR Sequence ID	Company	State	API® Bionumber	β galactosidase	Arginine Dihydrolase	Lysine Decarboxylase	Ornithine Decarboxylase	3Na Citrate (assim)	Hydrogen Sulfide	Urease	Tryptophan Deaminase	Indole Production	Acetoin Production	Gelatinase	Glucose	Mannitol	Inositol	Sorbitol	Rhamnose	Saccharose	Melibiose	Amygdaline	Arabinose	Oxidase	API® ID
LS18-3065-16	19	147	A	MS	4104002	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	Not valid, E.coli, Sal Gall. Hafnia alvei
LS18-3065-19	19	147	A	MS	4104002	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	Not valid, E.coli, Sal Gall. Hafnia alvei
LS18-3065-21	19	147	A	MS	4104002	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	Not valid, E.coli, Sal Gall. Hafnia alvei
LS18-3065-27	19	147	A	LA	4104002	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	Not valid, E.coli, Sal Gall. Hafnia alvei
LS20-4029-1.1	19	147	B	KY	4104012	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	Low disc Pullorum
LS18-2905-1.1	19	147	C	AR	4104110	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	Not valid Pullorum
LS18-2905-2.1	19	147	C	AR	4104110	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	Not valid Pullorum
LS17-2798-2.1	19	147	C	AR	4104112	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	+	-	Low disc Pullorum
LS17-2798-3.1	19	147	C	AR	4104112	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	+	-	Low disc Pullorum
LS18-3065-17	19	147	A	MS	4106002	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	Burkolderia cepacia 93%
LS18-3065-26	19	147	A	MS	4106002	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	Burkolderia cepacia 93%
LS20-4075-31	19	6	D	KY	6504752	-	+	+	+	-	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	Salmonella sp. 99.8%
LS20-4075-30	19	147	D	KY	6504542	-	+	+	+	-	+	-	-	-	-	-	+	+	-	+	-	-	+	-	+	-	Salmonella sp. 99.1%
LS19-3291-1.3	413	37	C	AL	6704752	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	Salmonella sp. 99.8%
LS17-2723-1.1	19	6	C	AL	6704752	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	Salmonella sp. 99.8%
LS17-2723-2.1	19	6	C	AL	6704752	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	Salmonella sp. 99.8%
LS20-4075-21	2379	36	D	Unk	6704752	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	Salmonella sp. 99.8%
LS20-4075-34	19	114	D	Unk	6704752	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	Salmonella sp. 99.8%

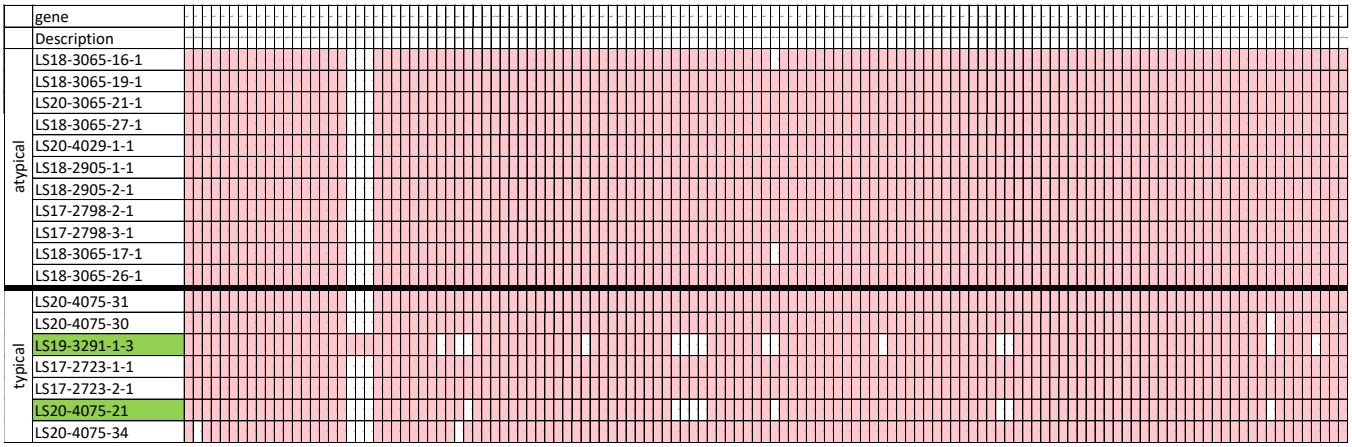
Yellow highlighted fields show biochemical differences between atypical and typical *Salmonella* phenotypes.

Table 2. Abricate heat map results of the Comprehensive Antibiotic Resistance Database (CARD).

			Atypical Bionumber											Typical Bionumber							
Database	gene	Description	LS18-3065-16-1	LS18-3065-19-1	LS20-3065-21-1	LS18-3065-27-1	LS20-4029-1-1	LS18-2905-1-1	LS18-2905-2-1	LS17-2798-2-1	LS17-2798-3-1	LS18-3065-17-1	LS18-3065-26-1	LS20-4075-31	LS20-4075-30	LS19-3291-1-3	LS17-2723-1-1	LS17-2723-2-1	LS20-4075-21	LS20-4075-34	Grand Total
card	AAC(6')-Iaa	AAC(6')-Iaa is a chromosomal-encoded aminoglycoside	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	acrB	Protein subunit of AcrA-AcrB-TolC multidrug efflux complex.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	acrD	AcrD is an aminoglycoside efflux pump expressed in E. coli.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	bacA	The bacA gene product (BacA) recycles undecaprenyl	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	baeR	BaeR is a response regulator that promotes the expression of	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	cpxA	CpxA is a membrane-localized sensor kinase that is activated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	CRP	CRP is a global regulator that represses MdtEF multidrug												1	1	1	1	1	1	1	7
	emrA	EmrA is a membrane fusion protein providing an efflux	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	emrB	emrB is a translocase in the emrB -TolC efflux protein in E.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	emrR	EmrR is a negative regulator for the EmrAB-TolC multidrug	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	Escherichia_coli_acrA	AcrA is a subunit of the AcrAB-TolC multidrug efflux system	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	Escherichia_coli_ampH	AmpH is a class C ampC-like beta-lactamase and penicillin-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	golS	GolS is a regulator activated by the presence of golD and	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	H-NS	H-NS is a histone-like protein involved in global gene	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	kdpE	kdpE is a transcriptional activator that is part of the two-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	marA	In the presence of antibiotic stress E. coli overexpresses the	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	mdsA	MdsA is the membrane fusion protein of the multidrug and	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	mdsB	MdsB is the inner membrane transporter of the multidrug	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	mdsC	MdsC is the outer membrane channel of the multidrug and	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	mdtB	MdtB is a transporter that forms a heteromultimer complex	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	mdtC	MdtC is a transporter that forms a heteromultimer complex	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	mdtK	A multidrug and toxic compound extrusions (MATE)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	msbA	MsbA is a multidrug resistance transporter homolog from E.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	ompA	OmpA is a porin that confers resistance to beta-lactam	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	ramA	RamA (resistance antibiotic multiple) is a positive regulator														1					1
	sdiA	SdiA is a cell division regulator that is also a positive	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	tet(B)	Tet(B) is a tetracycline efflux protein expressed in many																	1		1
	tolC	TolC is a protein subunit of many multidrug efflux complexes	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18
	yojI	YojI mediates resistance to the peptide antibiotic microcin	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	18

The presence of a particular resistance gene is highlighted in red, clear is absent. The two unique MLST isolates are outlined in blue. This table indicates the absence of the CRP gene in atypical *Salmonella* phenotypes.

Table 3. Abricate heat map of the Virulence Factor Database (VFDB).



The presence of a particular virulence gene is highlighted in red, clear is absent. The two unique MLST isolates are highlighted green. This table indicates no remarkable differences in the presence of virulence factors outside of the MLST distinctions.

Figure 1. Pangenome anlaysis of the core genomes.

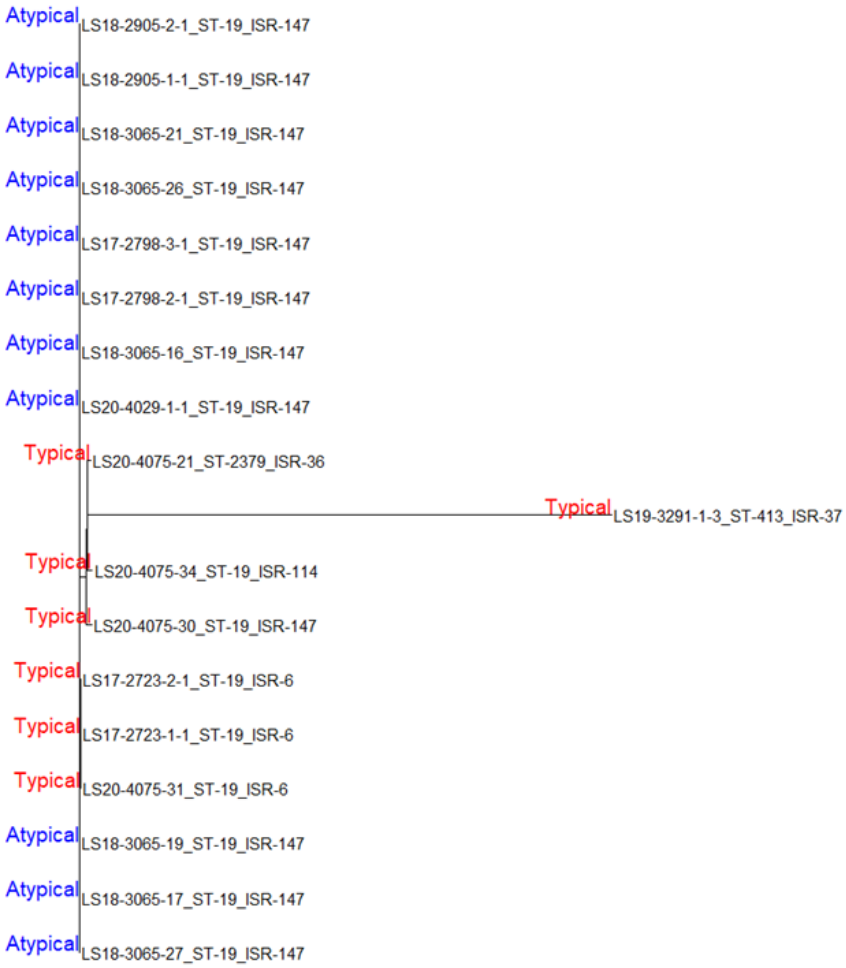


Figure 2. Pangenome analsysis of the accessory genomes.

Figure 3a. Principle component analysis of genomes.

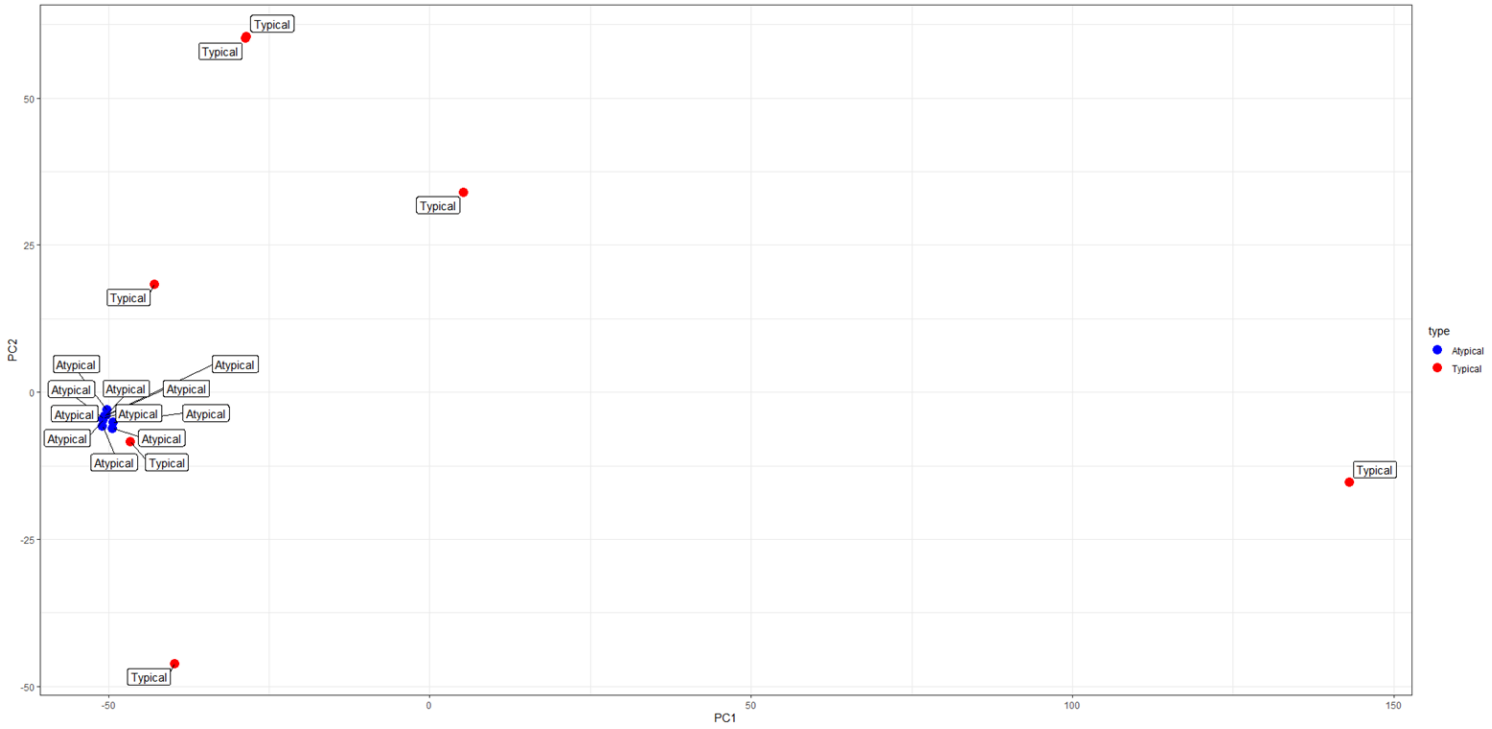
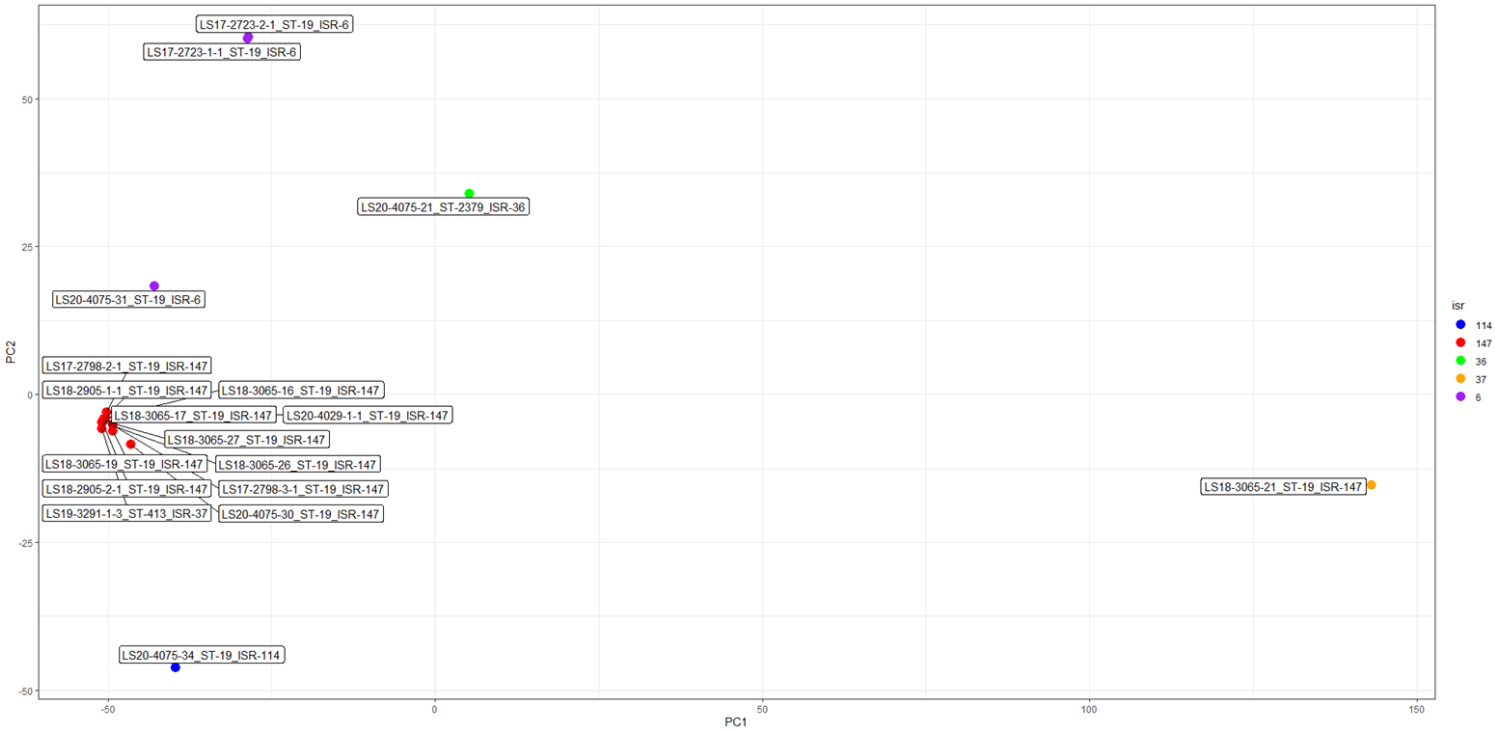


Figure 3b. Principle component analysis of genomes.



INFECTIOUS BRONCHITIS VIRUS SURVEILLANCE IN POULTRY FARMS OF THE CALIFORNIA CENTRAL VALLEY (2012-2020)

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SUMMARY

Infectious bronchitis virus (IBV) causes severe economic losses among chicken flocks worldwide. A constant active surveillance approach should be conducted to better understand the disease as well as target preventative strategies including vaccination and biosecurity. Even though IBV surveillance in broiler chickens has been performed for years, few analyses have been done to determine patterns and trends of IBV detection over time. The goal of this project is to understand seasonality and determine the predominant IBV genotypes over the years. Diagnostic laboratory reports from a single broiler company were compiled and analyzed. Case confirmation was based on positive RT-PCR and sequencing of the S1 hypervariable region. The data was analyzed using GIS and phylogenetic trees were used to compare S1 sequences. The distribution of IBV, seasonality and predominant genotypes are reported and discussed.

INTRODUCTION

IBV is a gammacoronavirus that induces mainly respiratory disease in chickens and causes severe economic losses among chicken flocks worldwide (1, 13). Coronavirus genetic diversity is due to point mutations, such as deletions and insertions, or recombination events that may occur during viral replication (12). Genetic diversity is also affected by live attenuated vaccination programs used in the poultry industry to control the virus. Continuously changing environments, such as seasonality and variation in dosage of IBV vaccines, provide opportunities for IBV variants to emerge (1, 3, 6). An active surveillance approach should be conducted to better understand the disease as well as to evaluate strategies including vaccination and biosecurity measures. Although IBV surveillance has been performed for years in California, few analyses have been done to determine the patterns of IBV detection over time (2,8). The purpose of the study is to provide an updated analysis of the infectious bronchitis surveillance system in California, to determine the temporal and spatial distribution of IBV, and to assess if seasonality is a contributing factor. In addition, the phylogeny of variant genotypes is also presented.

MATERIALS AND METHODS

IBV cases. The study design is a cross-sectional study looking at the poultry surveillance system at broiler chicken facilities in Central California (2). The cases were selected based on clinical signs and/or microscopic lesions consistent with IBV infections. A total of 4,765 chickens were sampled and submitted to one of the four California Animal Health and Food Safety (CAHFS) laboratories between January 2012 and January 2020. Of these, 1243 were tested by reverse transcriptase-PCR (RT-PCR) targeting the hypervariable region of the spike gene subunit 1 (S1) (7). The S1 gene has been used for genotyping and to determine the genetic diversity of IBV. From the 1243 RT-PCR-positive samples, 327 were sequenced. Sequences were analyzed using BLAST in GenBank to verify the variant genotype. Descriptive statistics was performed using SAS Studio, and the data was input as follows: 1 = positive IBV PCR and 0 = negative IBV PCR. Data generated included genotype, age, and number of birds per house and per farm. Seasonality were created based on the designated date recorded with some seasons not being reported due to missing data.

Spatial distribution of IBV. Using the city and county location data, we georeferenced all the commercial poultry farms within California (n=484) and performed a spatial analysis of chickens in premises using a density mapping analysis tool. Yearly heat maps were created using ArcGIS (online

version). The density analysis takes known quantities of chickens and spreads the numbers of IBV detections across the map to see where high concentrations of birds are located. Sites that had no IBV-positive birds on premises used the number of birds in a farm instead. The density map shows all points between 2012-2020, while heat maps were divided by year, showing the distribution of the chickens over time.

Spatial and temporal phylogenetic tree. The Nextstrain program was used to track the spatial and temporal evolution of genomic data of IBV sequences (n=321). Duplicates and unknown sequences were removed. Metadata (which includes the location, strain, and time the samples were collected) were combined with sequencing data. The sequences were aligned a phylogenetic tree was built, and the annotations with inferred ancestor pathogen dates were recorded (4). The trees were built using the maximum likelihood method. The ancestral state reconstruction of discrete traits such as county or region of isolation allowed the identification of possible transmission routes (9,11). In addition, we were able to infer the probable distribution of ancestor state at each node.

RESULTS

IBV genotyping. The strains identified in the study were California 99 (Cal 99), California 1737 (CA1737), California 3099 (CA 3099), California 115 (Cal 115), Massachusetts (Mass), Connecticut (Conn46), Iowa (IA 10705) and Arkansas (ArkDPI) strains.

Descriptive statistics. Of the 1243 cases that were tested by RT-PCR, 33.55% (417/1243) were negative while 66.45% (826/1243) were positive. Of the 327 IBV-positive samples that were sequenced, Cal 99 (n=103) and Massachusetts (n=71) strains had the highest frequency while Cal 115 and IA 107505 had the lowest (n=1) (Table 1). All sequences that were unidentified were classified as unknown due to improper annotations or missing data in GenBank. The effect of seasons did appear to be associated with IBV genotypes with a more pronounced peak in the spring (Figure 1); a logistic regression model with IBV status as outcome (reference level=negative for IBV) and seasons as exposure of interest (reference level=spring) with no adjustments. The odds ratios are all above 1, meaning that there is an association between seasons and IBV detection. The model shows that seasons are significant with an overall *P* value of 0.005. Chickens that tested PCR positive for IBV were 1.62 times more likely to be in the fall than spring. While chickens that tested PCR positive for IBV were 1.539 times more likely to occur in winter than spring. Both had significant *P* values of 0.0072 and 0.0032 respectively (Table 2).

IBV detection and broiler farm density. The heat map shows that the highest density of poultry farms in California is in the Livingston area from 2012-2020 (Figure 2). The heat map of each year shows the highest concentration fluctuates year to year, although it is primarily around the Livingston area. In addition, there are farms in the Fresno area with high concentrations of birds (Figure 3).

Phylogenetic tree. The phylogenetic tree shows the changing genomics of IBV in California over time. Figure 4 shows that Cal 99 (dark blue) strains were the most prominent from 2012 to 2014, but Massachusetts (light blue), CA 3099 (teal) and Conn 46 (light orange) became prominent by 2019. The tree line distance shows the number of mutations occurring and the parent-child relationship of strains (Figure 4).

DISCUSSION

Respiratory disease seasonality is of major importance in poultry production. It is thought that the incidence of respiratory diseases increase in winter, since the cold weather makes proper ventilation challenging and as a consequence the levels of ammonia are increased (10). In our study, statistical significances were observed in seasonality, with odds ratios above 1, which suggests that there is a difference between the seasons, especially when comparing spring to winter and spring to fall. This is mostly likely caused by spring having more PCR tests that were negative compared to fall and winter.

The descriptive statistics show that Mass and Arkansas strains present with the largest frequency in the affected chickens. In addition, a major shift in IBV genotypes was observed over the years, with Cal 99 being the most prevalent between 2012 and 2014 and Mass, Conn and CA 3099 taking over the IBV cases in 2019 and 2020. These temporal changes in IBV genotypes are likely due to the use of live-attenuated IBV vaccines in poultry in California, especially in the past few years. The introduction of live-attenuated vaccine strains, the mishandling of vaccines and inappropriate vaccine application may lead to rolling reactions and back-passaging of vaccine viruses within the flock (5), leading to the emergence of new variants.

High density farms such as those around Livingston are particularly vulnerable to having issues with IBV, especially when live-attenuated vaccines are being used on-farm or by neighboring farms. High density flocks that are heavily vaccinated provide opportunities for the virus to spread and mutate, allowing the virus to elude vaccination efforts.

In conclusion, increased biosecurity measures should be used in order to reduce the prevalence of IBV and transmission from farm to farm. Although vaccination is a valuable tool to prevent diseases such as IBV, it must be used with care and in association with other preventative measures, especially when using live-attenuated vaccines.

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Table 1. Frequency of infectious bronchitis virus genotypes diagnosed in broilers from California from 2012 to 2020.

Genotype	Frequency (n=327)	Percentage	Cumulative Frequency	Cumulative Percentage
ArkDPI	36	11	36	11
CA 1737	35	10.7	71	21.7
CA 3099	58	17.73	129	39.43
Ca 115	1	0.003	130	39.433
Cal 99	103	31.5	233	70.933
Conn 46	17	5.2	250	76.133
IA 10705	1	0.003	251	76.136
Mass	71	21.7	322	97.836
Unknown	5	0.15	327	100

Table 2. Logistic Regression of association between PCR diagnosis and seasonality in California from 2012 to 2020.

Seasons	Odds ratio	95% confidence interval		*P* value
Fall vs spring	1.612	1.138	2.284	0.0072*
Winter vs spring	1.539	1.155	2.051	0.0032*
Summer vs spring	1.068	0.742	1.536	0.7236

*Significant (P<0.05)

Figure 1. Frequency of infectious bronchitis cases reported in broiler farms in California from 2012-2020 (n=1243) divided by seasons.

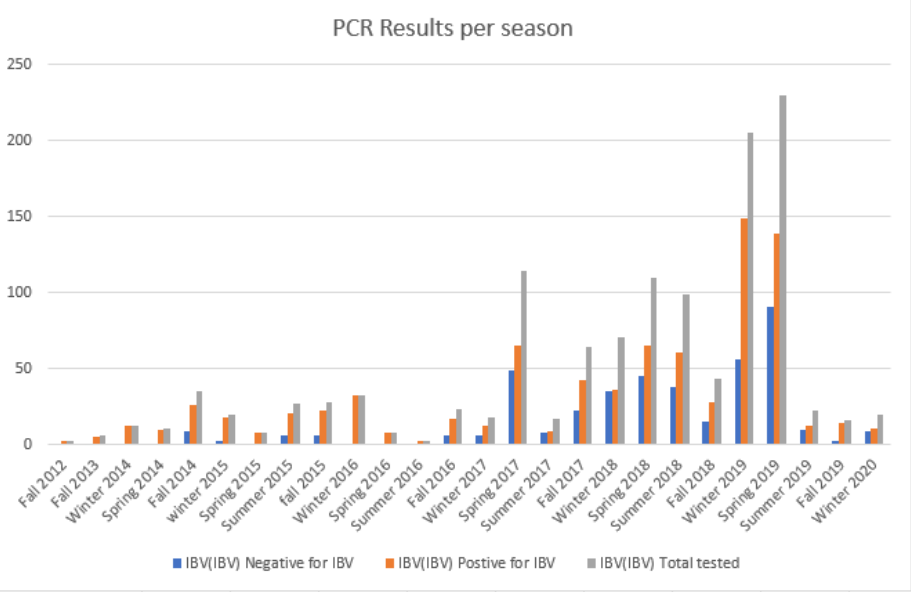


Figure 2. Heat maps representing the density of broiler chicken per farm in the Central Valley of California. The highest density is located in the region of Livingston CA (represented in yellow).

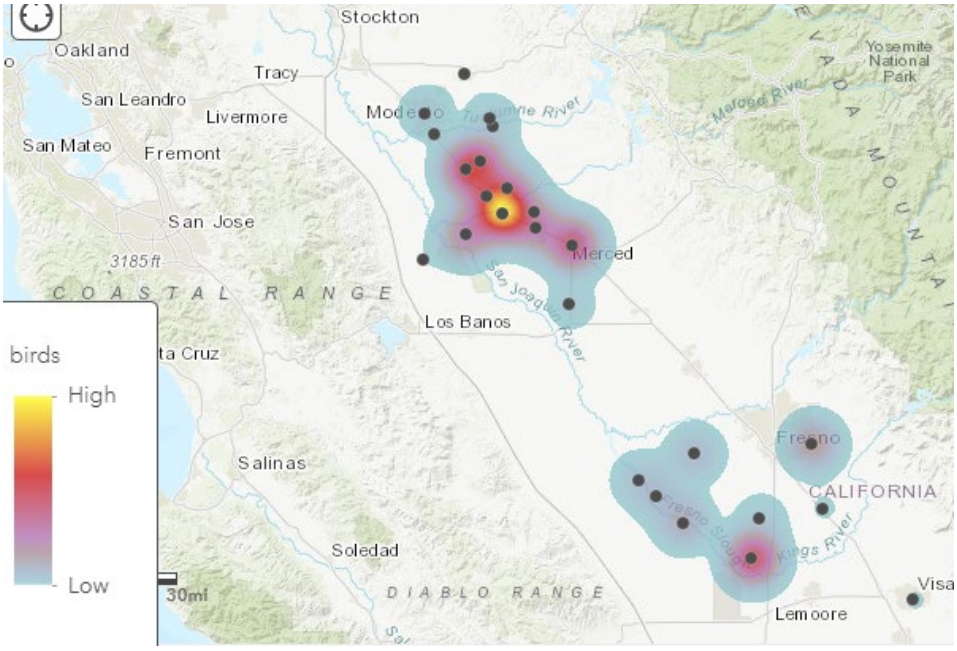


Figure 3. Yearly heat maps representing the density of broiler chicken per farm in the Central Valley of California. The highest density is located in the regions of Livingston and Fresno (at a lesser extent).

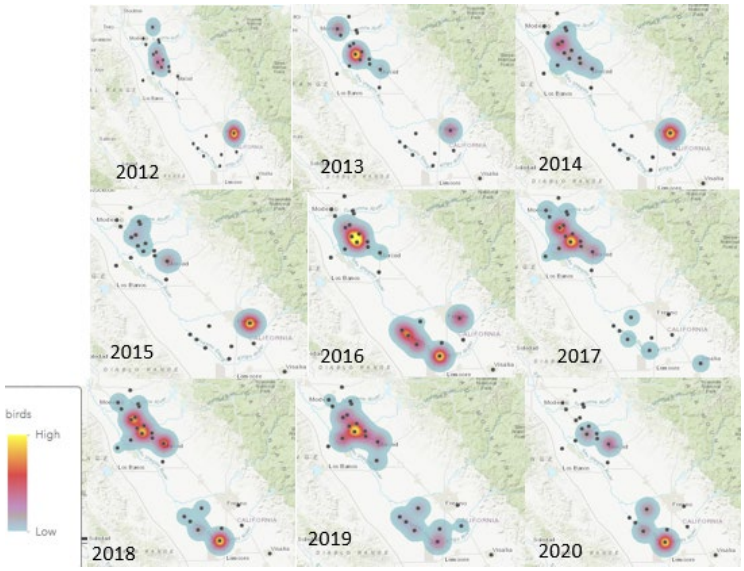
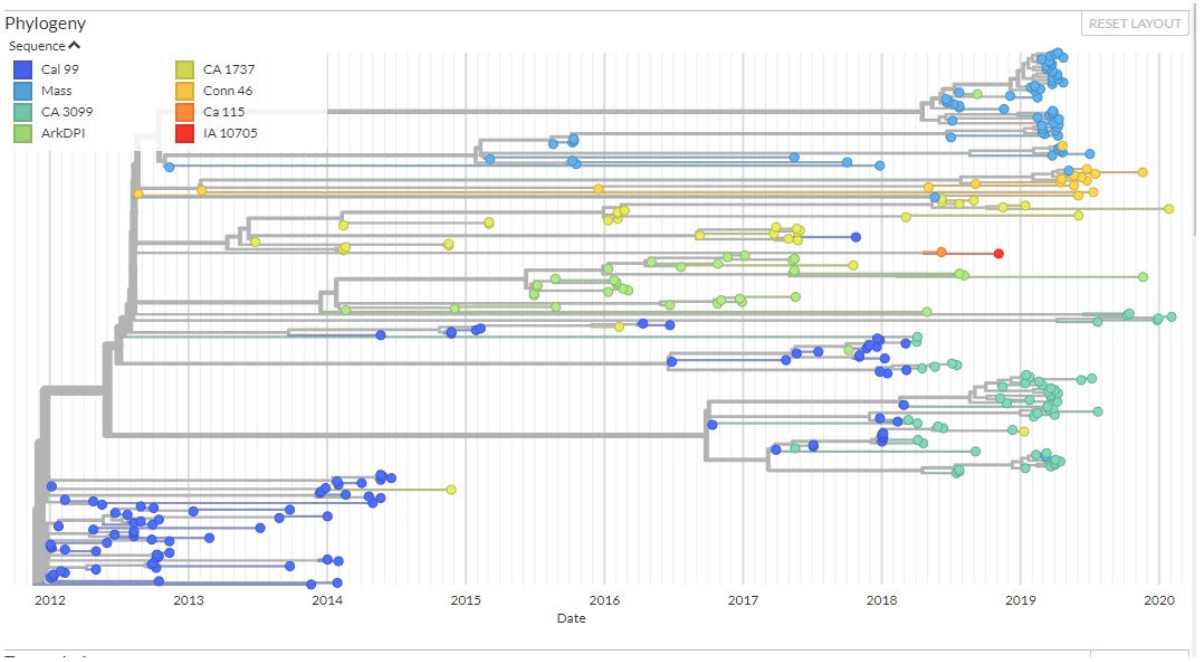


Figure 4. Temporal phylogenetic analysis of infectious bronchitis virus strains detected in California from 2012 to 2020.



USE OF A SPORULATED OOCYST COCCIDIOSIS VACCINE TO INDUCE IMMUNITY THROUGH LOW DOSE REPEATED FIELD APPLICATION UNDER PRACTICAL FIELD CONDITIONS

M. Mouw and D. Wilson

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SUMMARY

Coccidiosis vaccination of chickens is usually accomplished at the hatchery. Field conditions in commercial layer replacements are often not conducive to effective vaccine recycling and immunity development. An alternative vaccination strategy has been explored. Instead of depending upon natural recycling of oocysts, flocks are given a small dose of sporulated oocyst vaccine via feed at weekly intervals to simulate recycling, but without the requirement of access to feces or the appropriate environmental conditions for sporulation. The dose is lower than might be expected from natural recycling, but the ability of small doses of coccidia to stimulate immunity was described as "trickle infection" by Joyner and Norton in the 1970s. Successful protection of flocks using partial doses of vaccine at each interval (to achieve economic parity with a regular coccidiosis vaccination program) have demonstrated that protective immunity can be achieved without natural field recycling.

MATERIALS AND METHODS

- Continue Hatchery Application
- Trickle Dose
  - Multiple feed applications that will mimic fecal cycling
  - Sprayed directly on feed
  - 1/3 dose each applied at 7, 14, and 21 days of age
- Evaluation of Protection
  - Intestinal evaluation
    - Gross Lesions
    - Microscopic evaluation including enumeration and speciation
  - Challenge
    - Both vaccinates and naïve controls
    - 34x commercial Coccivac D2
  - Field Observation
    - Pullets moved to production facilities with a long history of clinical diseases

RESULTS

Vaccinated birds have shown protection thus far in the challenge as well as in the field.

ACKNOWLEDGEMENTS

Dr. Steve Fitz-Coy, Jerry Reeves, Dr. Jim Stockam

Figure 1. Intestinal Evaluation Day 28

Slide #	D	M	C	Comments
1	0	1miv	Gt	The parasite load was very light, most were rare to very few.
2	0	0	Gt	
3	1 bru	0	1ten Gt	
4	0	0	Gt hair	
5	0	0	1nec Gt	
6	0	0	1ten 1nec	
7	0	0	1ten Gt	
8	0	0	1 nec Gt	
9	0	LSFO	Gt	
10	0	0	Gt	

Challenge Microscopic Evaluation

Count of aliquot of challenge material ~ 1ml in vial
Total oocyst = 173,000/ml

Table 1: distribution of coccidia level in 10 immunized birds give Coccivac-D2

E .acerv/miv					*E. max*					*E. bru*					*E. ten*				
0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
7	3	1	0	0	8	1	1	0	0	10	0	0	0	0	10	0	0	0	0

Table 2: distribution of coccidia level in five control birds give Coccivac-D2

E .acerv/miv				*E. max*				*E. bru*				*E. ten*			
1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	2	2	0	2	1	0	2	0	0	3	0	1	1	0	0

PLANT-BASED SOLUTION COMPOSED OF CIMENOL RING HELPS TO CONTROL NECROTIC ENTERITIS IN EXPERIMENTALLY INFECTED BROILER CHICKENS

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SUMMARY

Cimenol ring is an active compound from plant extracts with strong bactericidal and fungicidal activities that works as a feed preservative and intestinal microbicide for farm animals. An experiment was conducted to evaluate its efficacy against necrotic enteritis (NE) with 480 broilers distributed into four treatment groups: non-infected control, infected control, infected birds with liquid cimenol ring in the water for 7 days after the infection, and infected birds with powder cimenol ring continuously in the feed. Infected birds received $2.5x10^8$ CFU of *Clostridium perfringens* (CP) on days 14, 15 and 16 of age. Performance, lesion scores and cecal counts showed that cimenol ring in infected birds could effectively control NE, whether as a preventive (powder) or as a treatment (liquid).

INTRODUCTION

Necrotic enteritis is an important disease in poultry due to the high economic losses in causes. Cimenol ring is an active compound from plants extracts with strong bactericidal and fungicidal activities (1) that proved to be effective against CP, the causative agent of NE, in laboratory and field trials. Previous studies also demonstrated cimenol ring's efficacy against other pathogenic bacteria and, also, that it is innocuous for the beneficial flora, which makes it a promising strategy to replace antibiotics in poultry farms. The aim of this study was to provide more information about the efficacy of the cimenol ring as a preventative and treatment for NE in experimentally challenged broilers.

MATERIALS AND METHODS

Animals. A total of 480 one-day-old Vencobb 400Y® broilers were weighted, wing branded and randomly distributed into four equal groups with 6 replicates of 20 animals each. They were raised for 40 days and provided feed and water *ad libitum*.

Treatment groups. "Non-infected control" (T1), "infected control" (T2); "treatment" group with infected birds with liquid cimenol ring in the drinking water at 1 ml/l for the 7 days after the infection (T3); and "preventive" group with infected birds with cimenol ring in powder at 0.5 kg/t of feed during all the trial (T4).

Challenge. CP was added to the drinking water through the drinkers at a rate to achieve $2.5x10^8$ CFU/bird on days 14, 15 and 16 of age in groups T2, T3 and T4. Water and feed were withdrawn for three to four hours prior to the challenge. Challenge water was consumed by the birds within one hour.

NE lesion scoring. Three birds per pen were humanely euthanized on day 17, necropsied and lesions were evaluated in a score from 0 (normal) to 3 (sloughed and blood small intestine mucosa and contents) (2).

Performance parameters. Body weight, weight gain, feed consumption and feed conversion ratio (FCR) were evaluated weekly. Morbidity was recorded daily. Efficiency index was calculated at the end of the trial as (survival rate x average final weight in kg x 100) / (days until slaughtering x FCR).

Enumeration of CP in cecal content. The cecal content was collected from the euthanized birds used for lesion scoring and samples were serially diluted to calculate mean CP CFU/g.

RESULTS

NE was declared on day 20 because of a mortality spike and the postmortem lesions of dead birds, therefore, the administration of liquid cimenol ring in T3 started on the same day. Weekly body weight was significantly influenced by the CP challenge and cimenol ring from day 21 until the end of trial. The groups with cimenol ring obtained significantly better weights than the others, with +158.4 g/bird as preventive (T4) and +118.89 g/bird as treatment (T3), compared to T2. No significant differences were observed in feed intake during all the trial. Cumulative FCR at the end of the trial was significantly better in the groups with cimenol ring,

compared to uninfected and infected controls. T3 (treatment) improved FCR by 11.1% and by 7.0% in T4 (preventive), compared to T2.

Efficiency index was 29.35 and 33.52 points better with cimenol ring as treatment and preventive, respectively, compared to T2. The supplementation of cimenol ring in water and feed significantly reduced overall mortality in the trial ($P<0.01$) compared to T2. The liquid supplementation (T3) also obtained significantly lower mortality rates than the uninfected control (T1). Mean lesion scoring was 0.00±0.00, 2.33±0.24 and 1.02±0.16 in control (T1), infected control (T2) and cimenol ring feed supplemented group (T4). T2 obtained significantly higher lesion scores compared to the control (T1) with lesions of NE in the small intestine and sloughed or bloody small intestinal mucosa and content. The group with cimenol ring as preventive (T4) also showed significantly higher lesion scores than T1 ($p<0.05$). However, unlike T2, bloody small intestinal mucosa and contents were not observed in T4. Enumeration of CP in cecal content differed significantly in T1, T2 and T4. T2 (infected control) obtained significantly higher counts, while T4 (preventative) obtained the lowest ($p<0.01$)

DISCUSSION

These results prove that cimenol ring is an effective solution to prevent and treat NE in broiler farms, as it obtained overall better productive parameters and lower mortality rates than infected birds.

NE prevention. Cimenol ring should be included in the feed continuously to prevent NE. It also helps to reduce intestinal lesion and decrease the number of CP colonies in cecal content.

NE treatment. As treatment, cimenol ring should be added to the drinking water as soon as NE is diagnosed for at least seven days, continuously. In conclusion, this experiment shows that cimenol ring can be considered a viable solution to replace antibiotics to treat NE in broiler farms.

ACKNOWLEDGEMENTS

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Figure 1. Weekly body weight, cumulative feed conversion rate, overall mortality and efficiency index per group.

Treatments	Weekly weight							Cummulative FCR	Overall mortality	Efficiency index
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 40	Day 40	Day 40	Day 40
T1 - Control	45.72 ±0.33	150.52 ±1.33	373.30 ±5.30	810.46^{a} ±10.71	1247.25 ±14.17	1686.65^{ab} ±24.59	1989.03^{b} ±26.52	1.96^{a} ±0.04	2.5^{c} ±0.5	247.36
T2 - Infected control	45.82 ±0.30	152.63 ±1.43	374.00 ±5.25	765.59^{b} ±9.84	1207.31 ±51.16	1616.36^{b} ±25.13	1971.99^{b} ±25.13	1.99^{a} ±0.04	10.83^{a} ±0.37	224.29
T3 - Liquid cimenol ring (treatment)	45.73 ±0.29	147.52 ±1.32	364.34 ±3.90	772.43^{b} ±9.46	1249.8 ±15.28	1703.99^{a} ±20.82	2130.39^{a} ±23.58	1.77^{b} ±0.03	6.66^{b} ±0.47	253.64
T4 - Powder cimenol ring (prevention)	45.53 ±0.29	150.67 ±1.48	377.73 ±4.91	811.83^{a} ±10.67	1244.79 ±15.03	1661.69^{ab} ±19.69	2090.88^{ab} ±22.07	1.85^{ab} ±0.08	3.33^{bc} ±0.47	257.81
Significance	NS	NS	NS	P<0.01	NS	P<0.05	P<0.01	p>0.05	p<0.01	-

NS: Not significant. a, b, c: mean values with the same superscript within a column do not differ significantly ($P<0.05$).

Figure 2. Mean number of CFU of CP per gram of cecal content of birds from different treatments (n=6; 3 samples x 2 dilutions).

Treatment	CFU of *C. perfringens*/g
T1 – Control	0.02 ± 0.01^{c}
T2 – Infected control	3.59 ± 0.50^{a}
T4 – Powder cimenol ring (prevention)	1.31 ± 0.29^{b}
Significance	P<0.01

a, b, c: mean values with the same superscript within a column do not differ significantly (P<0.05).

ARE YOUR POULTRY GOING PECKING CRAZY? ENRICH THEIR ENVIRONMENT WITH THE RIGHT TOOLS

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SUMMARY

Poultry often use their beak like a hand for positive actions such as foraging and aggressive actions such as injurious pecking. Aggressive behavior in poultry occurs due to a multitude of factors that are linked to areas such as genetics, rearing, nutrition and the environment. The reason behind these aggressive behaviors have been debated in research relating to unfulfilled behaviors that occur in the natural environment, and environmentally induced neurobiological alterations. In the field, this aggression can appear and spread through a flock creating performance issues and injuries. Combating these behaviors through various means, and providing environmental enrichment, help to re-direct aggression and promote positive behaviors. Environmental enrichments can be varied but can include blocks which provide pecking and perching opportunities and help avoid frustration. Reviewing positive and aggressive behaviors with actions to encourage or mitigate, respectively, is a developing yet important component to clinical poultry veterinary practice.

INTRODUCTION

Animal welfare is a broad topic that covers many aspects of animal wellbeing which can include both the health and the natural behaviors of the animal. Often when animal welfare is discussed the five freedoms are usually at the forefront of the conversation and they include: 1) freedom from hunger and thirst; 2) freedom from pain, injury and disease; 3) freedom from distress; 4) freedom from discomfort; and 5) freedom to express behaviors that promote well-being (1). While the five freedoms have been criticized for focusing on the negative aspects of welfare; the opposite can be true in that if the five freedoms are sufficiently met, then both the health and natural behavior welfare of the animal is satisfied (2). The health of the animal is critical; however, for this review the focus will be on encouraging natural actions of poultry to reduce negative behaviors that may have an indirect impact on the overall health of the bird.

Commercial poultry are often reared in barren environments (3) with some exceptions such as layers in alternative housing, adult broiler breeders and some organic production styles. This type of production environment has been used because it has provided a successful means to raise poultry; however, this barren environment may lead to boredom, fear and often abnormal harmful behaviors, such as excess aggression or feather pecking (3, 4). Feather pecking is often associated with layers or broiler breeders but can also be head pecking aggression in turkeys or in older broilers as general aggression (4-7). Feather pecking is an injurious behavior where the birds peck at, eventually remove, and may consume feathers of other birds in the flock which can result in plumage and skin damage (4). Feather pecking is thought to be a redirected behavior that may stem from frustration at lack of foraging and feeding opportunities (4).

Combatting feather pecking in addition to boredom, fear, and other abnormal behaviors due to barren environments require a multifactorial management approach, but one of the main strategies used is environmental enrichment. Newberry (8) defined environmental enrichment as the modification of the environment of captive animals which supports an increase in the animal's behavioral possibilities that can lead to improvements in biological function. In order for the enrichment to be effective it has to be biologically relevant to the animal and serve four major purposes: 1) increase the occurrence and range of the animal's species specific behavior; 2) prevent the development of abnormal behavior or decrease the extent and complexity of this behavior; 3) increase positive exploitation of the environment; and 4) increase the animal's ability to handle behavioral and physiological challenges (9). For the environmental enrichment to be deemed successful there are four criteria that have to be met: 1) increase species specific behavior; 2) maintain or increase the levels of health; 3) increase the economics of the production system; and 4) be practical to employ (9,10). The challenge with environmental enrichments and use across all poultry is that different types of poultry have different behavioral expressions, housing and management criteria.

LAYERS

The concept of environmental enrichment has been very prominent over the last several years in layer

production due to public conversations on different housing systems. Most of the focus on enrichment in layer production has concentrated on the adult system, but with increasingly complex housing systems being used commercially, research has started to look at the impact on the rearing environment on long-term behavior (11).

In adult layer hen housing systems, conventional housing has been shown to restrict certain behavioral expressions but have been beneficial for the health of the bird (11,12). Conversely, alternative systems allow for these natural behavioral expressions but may increase incidences of skeletal injuries, mortality and feather pecking (11,12). The negative behavior of feather pecking has been shown to affect between 8 to 65% of flocks and depending on internal and external factors between 15 to 95% of the birds within the flock can be impacted (4). Most layer hens in Canada and the USA, regardless of the system they are reared in, have gone through beak trimming to dull the end of the beak to reduce injury from feather pecking. While the application of this practice has improved with advances in technology, there are still health risks to the bird and the feather pecking behavior is not changed but rather the consequence of the behavior is reduced (4). Additionally, the practice of beak trimming has faced increased negative publicity due to societal concerns (4). Van staaveren *et al.* (4) completed a meta-analysis and reviewed three environmental enrichment categories over different housing types, age and beak trimming practice as they related to feather pecking and damage: 1) materials for foraging and consumption of feed; 2) materials for dust bathing; and 3) materials with no foraging or dust bathing opportunities. Feather pecking and resulting feather damage was found to increase in frequency as the flock got older (4). Contrary to previous research, there was a general increase in feather pecking and feather damage in birds without access to enrichments and for those in conventional cages (4, 11). Provision of enrichment *per se* was significantly associated with lower feather pecking (4). However, the type of enrichment that was the best could not be determined (4). The researchers suggested that those enrichments that give birds opportunities to forage and to a lesser extent dustbathe were most effective (4). Since feather pecking and resulting feather damage is multifactorial in origin, multiple management solutions should be applied to deter this negative behavior and decrease its impact.

Rearing layers has seemed to become more of a focus commercially as alternative systems have been increasingly employed. In the field, it is often noted that layer pullets do best in their adult environment if they have been reared in a similar environment when they were young. Research settings have found that enriching rearing environments with physical, sensory and stimulatory objects may support visual development, neurobehavioral development, auditory stimulation, skeletal development, immune function, and behavioral development (11). While there may be limited commercial studies on these factors, the research appears to support what has been noticed anecdotally in the field. For example, when pullets destined for complex alternative housing environments are reared in complex environments, they often settle into their new environment better than pullets reared in different or more barren environments. In the context of feather pecking, which can be a major issue in adult hens, a chick generally starts foraging behavior within the first week of life and without any foraging material this can turn into damaging behavior by 4 weeks of age (11). A pullet environment can be enriched to prevent or re-direct this behavior and has been shown to have a significant impact on reducing the incidence of feather pecking during rearing or laying (11).

BROILER BREEDERS

Similar to the challenges in layer hen rearing and transition to alternative housing, the young breeder has to transition from a two-dimensional world (often being on litter to find feed and water) to a three-dimensional space in the breeder facility (having to jump on and off slats for food, water, and move into nest boxes) (5). In breeders both the males and females would have increased stress during the transition from rearing to breeding. For females, the stress comes from navigating the new environment and interaction with aggressive males; whereas with males, the stress often originates from male competition (5, 13). Additionally, during rearing the needed feed restriction to ensure fertility later in life presents another area of stress in breeders that has been linked with performing behaviors indicative of frustration, boredom, and hunger (14).

In both the rearing and the breeding phase, environmental enrichment can support a larger range of behavioral choices that can have a positive impact on both health and behavioral welfare issues (14). With the transition from the rearing to the breeding facility it is important that the birds have access to environmental enrichments that support their learning of a three-dimensional environment such as perching objects and slats (5). Early access to these enrichments is critical for brain development and formation of long-lasting cognitive skills that influence navigation, use of space, fearfulness and learning (5). In older breeders, access to perches and covers have shown encouraging benefits on the space use of males,

reduced aggression in males, reduced fear responses in females and improved fertility and hatchability (13).

Environmental enrichments in breeder flocks during rearing or breeding should be multifactorial because of the multiple stresses that breeders must contend with, such as feed restriction and the need to learn spatial navigation. Diverse environmental enrichment programs have been shown to reduce the incidence of feather pecking and feather damage in young breeders as well as decrease over mating (5). Effective enrichment should offer positive reinforcement such as food or cover to have lasting effects (5). When birds are feed restricted enrichments that offer foraging and dust bathing opportunities have shown positive impacts on reducing repetitive pecking and aggression. However, these resources are best used in a combination strategy with other enrichments (14).

BROILERS

Behavioral challenges in broilers are not often discussed because, in general, fast-growing broilers are not cannibalistic and are less aggressive than other poultry (7). Often the behaviors that are associated with young birds and, consequently broilers, relate to play behaviors and general use of space (7,9). Play behaviors are common in young animals and is not critical to survival, occurs when stress is low and can occur repetitively, but not stereotypically (7). In chickens, these play behaviors may be frolicking (spontaneous running with raised or flapping wings), sparring (play fighting with minimal physical contact) and worm/food running (bird picks up object and runs while being chased by others) (7). While there has been no direct association with environmental enrichment and play behaviors in broilers, these enrichments may encourage other positive behaviors such as better use of space and foraging (7,9,15). When designing and using environmental enrichments for broilers it is important to keep in mind the size and body conformation difference from other poultry. For example, broilers would prefer raised platforms over round perches (9). Additionally, it is important to provide the enrichment in an area that is used for activity rather than resting and has higher light intensity (9). In commercial fast-growing broilers, the enrichments may start to be used around one week of age and often starts to decline in use around three to four weeks, but can last to six weeks, especially if the activity is perching (9).

TURKEYS

Scientific literature on turkey welfare is a small but growing field. Some of the main factors affecting turkey behavior and welfare are stocking density, space availability, spatial distribution, aging, lighting, feeding and transport (6). Similar to broilers, there appears to be a decline in overall activity as well as a reduction in feeding, foraging, drinking, preening and pecking as turkeys age. However, as turkeys became closer to sexual maturity increased activity relating to mating behavior occurred (6). Interestingly, turkeys have not been shown to dust bathe or ground scratch as is seen in chickens, but both running and frolicking have been observed (6). Additionally, the incidence of feather pecking and cannibalism increased with age in turkeys, yet some researchers have found damaging pecking as early as the first or second week of age (6,16). In turkeys, head pecking is an act of aggression while other research suggests that severe feather pecking (non-social) is re-directed ground foraging behaviors (17). Often research has suggested that in turkeys, similar to other poultry, a lack of suitable environmental features re-directed natural behaviors to become negative and stereotypical (17). Since negative pecking behaviors can be learned and observed early in young poults, some researchers have suggested that foraging objects used to re-direct pecking may have a stronger effect when provided during brooding as a preventative measure (17,18). However, turkeys may also express pecking and aggressive behaviors after an environmental disturbance to restore pre-existing dominances and address unfamiliar individuals, especially in males (16). Severe pecking and aggression are often multifactorial in origin and should consider genetics, nutrition and the environment (17). Preliminary research in turkeys suggests that environmental enrichments chosen by the bird, are new to the flock and provide positive feedback can discourage injurious pecking (17,18).

FORAGING BLOCK

Many different types of environmental enrichments can meet the four major purposes and criteria for success as described previously in the introduction section. There are multiple environmental enrichments that are practical and can provide more than one opportunity to complete multiple behaviors which support the economics of the production system. A unique example of this is a foraging block that contains whole grains, minerals and has a sweet scent (19). The contents and construction of the block attracts the bird and then provides a reward as the bird can remove pieces and grains (Figure 1). Additionally, the size of the block allows poultry to jump on the block. Depending on the age and size of the birds a single or several birds can perch on the flat, raised surface at a time (Figure 2). Anecdotally, the block has

served to re-direct negative pecking and aggression behavior in young and adult breeders, turkeys and layers (20). Additionally, the block has anecdotally served to support additional positive behavior opportunities in broilers such as perching on top and around the block as well as pecking at the block (20).

CONCLUSION

There are common themes across different poultry sectors in relation to welfare needs and supporting positive behaviors, yet each poultry type has some unique features that must be taken into consideration. For example, behaviors of layers and breeders are similar in the sense that they both need to adapt to changing environments when they move from the rearing to the adult barn, but many differences exist in terms of management practices. Additionally, there are differences in behaviors that are expressed if the bird is younger versus older and the physical size the bird reaches. A commonality among most poultry is the negative behaviors surrounding aggression and stereotypic pecking (re-directed foraging). Encouraging positive poultry behaviors and re-directing stereotypic behaviors such as injurious pecking is multifaceted and requires an approach that encompasses many factors such as management, nutrition, health, genetics, and environmental enrichment. When an environmental enrichment is chosen is it important that it is biologically relevant for the animal and commercially practical.

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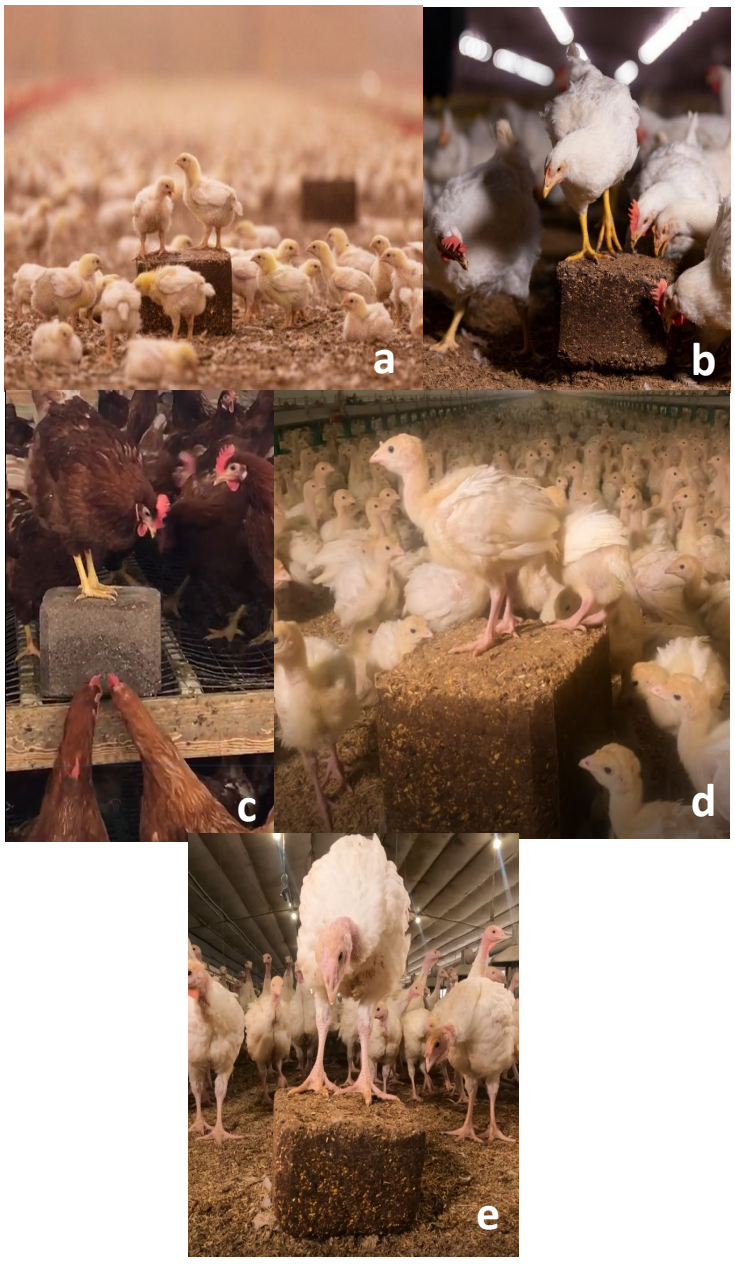
Figure 1. Assorted pictures of broilers [Canada production] (a), broiler breeders [USA production] (b,c), layers [USA production] (d) and turkeys [USA production] (e,f) pecking at the foraging block.

Figure 2. Assorted pictures of broilers [USA production] (a), broiler breeders [USA production] (b), layers [USA production] (c) and turkeys [USA production] (d,e) perching on the foraging block.



A RETROSPECTIVE STUDY TO ESTABLISH BASELINE DATA FOR *MYCOPLASMA GALLISEPTICUM* POSITIVE COMMERCIAL TURKEYS IN CALIFORNIA: 2008-2019

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SUMMARY

Mycoplasma gallisepticum (MG) is an economically significant pathogen of poultry which results in severe disease especially when complicated by other respiratory pathogens. The objectives of this study, were to establish preliminary baseline data on the respiratory pathogens in MG positive commercial turkey flocks in California and to determine the age of clinical disease presentation. The retrospective study analyzed 54 flocks in which MG was confirmed in central California from 2008 to 2019 by the California Animal Health and Food Safety (CAHFS) laboratory system. Of the 54 flocks that were confirmed positive by real time polymerase chain reaction (rPCR), 34 (63%) had positive MG ELISA titers and 20 (37%) were negative. *Escherichia coli* was the most common pathogen isolated; flocks followed by *Bordetella avium, Mycoplasma synoviae* and *Ornithobacterium rhinotracheale* respectively. The mean age at which flocks were submitted for necropsy to the CAHFS laboratories and confirmed as MG rPCR positive was approximately 15 weeks. This study established preliminary baseline that may aid in the implementation of targeted control and prevention strategies for California's turkey industry.

INTRODUCTION

Mycoplasma gallisepticum (MG) is considered the most economically significant mycoplasma pathogen in poultry globally (1). The effects of MG in poultry includes chronic respiratory disease, reduced feed efficiency, decreased growth, lowered egg production and increased carcass downgrades and condemnations (1). Turkeys (*Meleagridis gallopavo*) are more susceptible to MG than chickens, commonly developing more severe chronic respiratory clinical signs including conjunctivitis and sinusitis. Although classified as a primary pathogen, MG-associated morbidity and mortality is exacerbated by concurrent infection with other respiratory pathogens, such as *Escherichia coli* or when complicated by environmental stressors (2,3,4).

California is one of the top ten turkey producing states in the US (5). There is limited published epidemiological studies on the potential effects of MG in commercial turkey operations or the involvement, effect(s), and correlation of other co-respiratory pathogens on disease incidence. In this retrospective study, MG rPCR positive cases in California commercial turkey flocks submitted to the CAHFS laboratory system were analyzed over a 12-year period (2008-2019). Case submissions in the CAHFS database were analyzed to identify the most common bacterial and/or viral co-pathogens involved in MG positive turkeys as well as the mean age of detection. Results can be used to better understand concomitant MG based outbreaks and facilitate control and prevention strategies.

MATERIALS AND METHODS

Selection of cases. The CAHFS laboratory system electronic database (SLIMS v.10) was searched for all turkey flock submissions with a positive MG diagnosis by rPCR between the years 2008 to 2019. The diagnostic test methodologies used for MG detection and surveillance at CAHFS are in accordance with the National Poultry Improvement Plan (NPIP) and include enzyme linked immunosorbent assay (ELISA), hemagglutination inhibition (HI), serum plate agglutination (SPA), and real time polymerase chain reaction (rPCR), with rPCR being the main confirmatory test used in poultry necropsy submissions (6). Only flocks with complete records of predictor variables and confirmed as MG positive based on rPCR were included in this study

analysis. The serological data was evaluated classifying the rPCR positive flocks as seropositive or negative depending on titer groups and values as described below.

Detection of respiratory pathogens. The respiratory pathogens detected in flock submissions in addition to MG, were identified by diagnostic tests available at CAHFS and test modalities included bacterial culture, fungal culture, histopathology, PCR, and serology (7,8,9).

RESULTS

Descriptions of submissions. A total of 54 commercial turkey flocks were included in our study after they were confirmed as MG positive by rPCR. Birds from these flocks were submitted to the CAHFS laboratory for necropsy evaluation due to increased morbidity and mortality. The 54 flocks represented three vertical integrated commercial turkey producers and a total of 22 premises.

Flocks. rPCR confirmed MG positive turkey flocks were between 6 to 50 weeks old with a mean age of approximately 15 weeks and median of 13 weeks. The turkey flocks included 51 meat type flocks and three breeder type flocks. Sex was evenly distributed among the cases.

Co-pathogens. *Escherichia coli* was the most common pathogen, followed by *Bordetella avium, Mycoplasma synoviae*, and *Ornithobacterium* rhinotracheale, respectively. *Aspergillus*, *Staphylococcus aureus*, *Histomonas meleagridis*, and *Riemerella anatipestifer* were identified in a few samples.

DISCUSSION

The mean age at which flocks were submitted for necropsy to the CAHFS facility and confirmed as MG positive was approximately 15 weeks. This finding is salient as experimental studies in turkeys have shown clinical signs such as sinusitis develop 6 to 10 days after inoculation with MG (2). These data suggest that exposure may have taken place between weeks 13 to 14. Therefore, increased levels of biosecurity and good management practices during this period may help in controlling and preventing transmission by reducing environmental stressors.

MG infections must be differentiated from other respiratory diseases, as the severity of disease is influenced by other respiratory pathogens (2,3,9,10). Chronic respiratory disease (CRD) describes a severe airsacculitis that is the result of MG or MS infection complicated by respiratory pathogens, usually respiratory viruses and *Escherichia coli* in an intricate disease complex (2,4,10). In this study, *Escherichia coli, Bordetella avium, Mycoplasma synoviae*, and *Ornithobacterium rhinotracheale* were the main concurrent pathogens identified in MG positive commercial turkey flocks. These findings are useful in the development of control and prevention strategies since pathogens such as *Escherichia coli* are often linked to poor farm management practices (3). Furthermore, other pathogens such as MS have a negative synergistic effects alongside MG and are more likely to occur when immunosuppressive agents such as hemorrhagic enteritis virus is present (11,12). There is no definitive test or method to determine the order of occurrence of these pathogens at the farm level.

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

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COMPARATIVE VACCINATION EFFICIENCY AGAINST INFECTIOUS BURSAL DISEASE IN COMMERCIAL LAYERS

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SUMMARY

This study's objective was to compare the vaccination efficiency of a novel immune-complex (ICx) vaccine against infectious bursal disease (IBD) virus in commercial layer flocks compared to alternative IBD vaccination methods. Two different field studies were conducted in commercial layer operations involving a total of 197,425 birds using separate poultry houses for each treatment in the same farms and similar management conditions. The first field study compared the protection against IBDV field challenge by either ICx or rHVT-IBD Gumboro vaccines and the second study in a different commercial layer operation compared the protection given by the ICx at the hatchery vs. field vaccination via "oral drop" at 12 and 18 days of age. Parameters measured in both studies were serology, histopathology, PCR for IBDV detection and sequencing, body weight, feed intake, mortality, and uniformity of the vaccinated flocks. The vaccine takes, and seroconversion titers were higher in ICx vaccinated groups with lower mortality and histopathological lesion scores that means the novel ICx vaccine is safe and provides proper protection against IBD.

INTRODUCTION

Although IBD was identified more than 50 years ago (1), it continues to be a significant threat to commercial poultry an one of the most critical poultry diseases worldwide (2), IBD also known as Gumboro disease, is a highly contagious, immunosuppressive disease of young chickens (3,4). The Infectious bursal disease virus (IBDV) is caused by a *Birnavirus* (5), with two different serotypes (serotypes 1 and 2) have been described, and antigenic variants of both serotypes have been recognized. However, only viruses of serotype 1 are pathogenic. The economic importance of this disease is manifested in two ways. First, some virus strains cause high morbidity and mortality, up to 60% in laying hens and 25% in broilers 3-weeks of age and older (4). The second manifestation is the immunosuppressive effect of IBD diminish bird's resistance to other diseases and lessen response to common vaccines administered against other pathogens. For the reasons mentioned above, IBD is one of the most economically significant diseases (6).

IBD is a permanent risk in layer-type operation; according to Van den Berg (4), the layers are usually more susceptibles to the IBDV than meat-type birds. The disease control is based on strict compliance with rules of hygiene and disinfection in addition to vaccination. Classical serotype 1 vaccines still induce adequate protection, but the actual problem for controlling the disease is MDA interference in establishing the vaccination schedule (7). MDA is the first line of defence of layer pullets during the first weeks of age; however, the decay of MDA in this type of bird is slow and uneven, complicating the vaccine's decision to use and the day of application (8). Nowadays, oil-emulsion inactivated virus, attenuated live virus, recombinant and ICx vaccines are commercially available to protect birds against IBD (9); until now, to our knowledge, there was no ICx vaccine developed explicitly for layers available in the market. This study was conducted to evaluate the performance of the ICx vaccine under commercial conditions.

MATERIALS AND METHODS

Two different trials were conducted in different layer commercial companies involving a total of 197,425 birds to compare an ICx vaccine's vaccination efficiency against IBD. The first study compared the protection against IBDV challenge by either ICx or rHVT-IBD vaccine as a single dose at the hatchery, and in the second trial, the comparison was between the ICx vaccine at a single shoot at the hatchery versus

field vaccination by oral administration at 12 and 18 days of age. In both cases, the birds were reared in separate houses according to their vaccination program. As much as possible different management practice as gradings, vaccinations, and feed changes were performed in the same age for control and treatment groups. Parameters measured in both studies were serology using an Idexx conventional test, histopathology, PCR-RFLP for IBDV, body weight, feed intake, mortality, and uniformity of the vaccinated groups.

RESULTS

The PCR results in trial one were as follows for the group vaccinated with the ICx vaccine (strain SYZA 26); in this case, the strain was recovered 80% of the time, while in the group vaccinated con rHVT IBD product, 90% of the time the variant strain was detected. In trial two, the SYZA 26 was recovered in 87% of the cases compared to 73% recovery in the group with two doses of live attenuated vaccines. The serological response was also demonstrated, showing higher geometrical mean titers and a lower coefficient of variation at the end of the rearing period in the ICx group for both trials. According to Rosales (10), the bursa lesion score was performed; for trial 1 the rHVT IBD showed higher damage lesion, confirming that this kind of vaccine does not protect the bursa damage generated by the IBDV field strains (2). In trial two, no difference was found in the average of the lesions detected in the bursa, which means that the bursa lesion is not different from what is seen with the available attenuated vaccines. Performance data were analyzed using one-way ANOVA with a $P<0.05$ level of significance, cumulative mortality in trial 2 was lower in ICx treatment ($P=0.017$) in comparison to the control group. Bodyweight in trial one was higher at fifteen weeks in favor of ICx treatment ($P=0.048$). There were no differences between the treatments on uniformity or cumulative feed intake.

CONCLUSIONS

In conclusion, the ICx vaccinated flocks showed the best seroconversion in the presence of MDA: Effective IBD vaccination was observed in the ICx groups, as the results indicated vaccine strain detection over 87% by PCR in both trials. Vaccination with rHVT-IBD vaccine demonstrated the field IBDV strain, as PCR detected it in 90% of the samples and showed a variant E of IBDV. Higher histopathological lesions were reported in the bursas evaluated in this group. The vaccination efficiency of IBD field vaccine administration in trial two was unable to allow proper induction of immunity against IBD, showing 27% of the samples were found negative by PCR. At the end of the rearing period, Elisa's (Idexx) seroconversion showed higher mean titers and a lower coefficient of variation. A lower mortality rate was found in the ICx vaccine group, and no difference was observed in feed intake neither uniformity. Therefore, using an ICx vaccine strain, Syza 26, confers optimal protection against IBD and fulfills the most critical criteria on safety, efficacy, and adjustment to the MDA decay.

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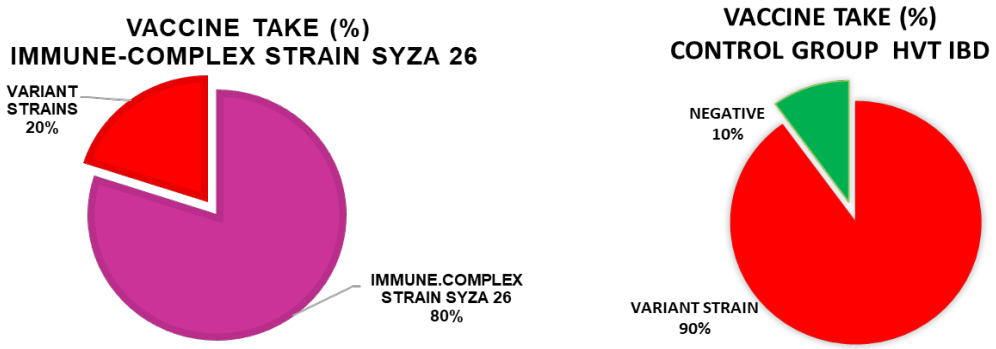
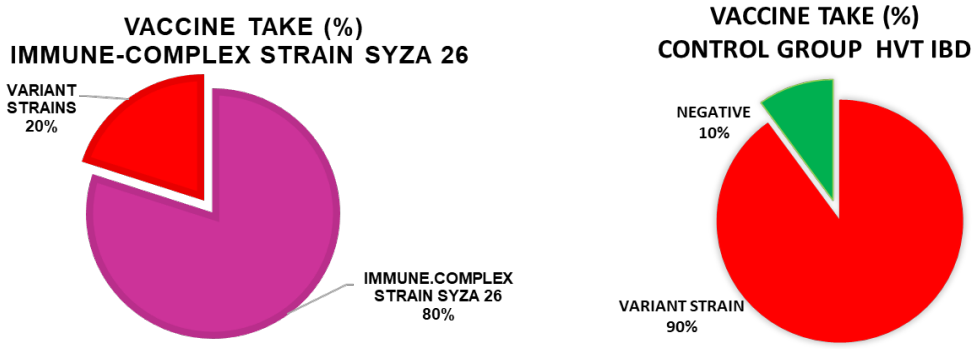
Figure 1. Results of trial 1: vaccine strain detection according to the vaccination program.



Results of trial 1: Vaccine strain detection according to the vaccination program.

Figure 2. Results of trial 2: vaccine strain detection according to the vaccination program.



Results of trial 1: Vaccine strain detection according to the vaccination program.

DON'T LET MYCOTOXINS RUIN YOUR GOOD EGGS

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SUMMARY

A common myth is the belief that mycotoxins appear alone when contaminating a grain, but a single grain can have several mycotoxin families. In finished feed it is possible that multiple mycotoxins may be present. Realistically, low- to medium-level challenges may be found in finished feed and impact rapidly dividing cells found in areas such as the immune, intestinal, and reproductive system. Poultry sensitivity to mycotoxins can vary but mycotoxins have been shown to play a role in bird health and performance. In birds such as broiler breeders, realistic mycotoxin contamination doses may decrease egg production, decrease shell quality, and increase embryonic mortality without having obvious effects on the breeder. Subclinical mycotoxicosis is thought to be a common occurrence in production but diagnosis can be difficult. Understanding the direct and indirect impacts of mycotoxins, in addition to testing, help to better diagnose the issue.

MYCOTOXINS: A PROBLEMATIC PUZZLE

Mycotoxins are natural secondary toxic metabolites produced by fungi that can be found in crops and feedstuffs such as cereal grains and their by-products. These compounds can negatively affect people and livestock through direct and indirect effects(1, 2). The presence of these metabolites can occur prior to harvest, but they can also occur at any point after harvest, transport, and storage. Once these metabolites are formed, they are very chemically stable and continue to persist in the contaminated ingredient even after becoming a finished feed.

In addition to the typical stressors that are associated with intensive rearing practices associated with poultry production systems, birds, particularly chickens and turkeys, can be susceptible to a wide array of mycotoxins. Their effects can vary depending on multiple factors such as species, health status, age, exposure time, type of exposure and load of exposure. Poultry are exposed to a variety of mycotoxins via feedstuffs and bedding materials. In poultry feed, mycotoxicosis was thought to be associated to the exposure to a particular mycotoxin; however, contamination with multiple types of mycotoxins on a single feedstuff source tends to occur more frequently. As a result, commonly used ingredients such as corn, soy, and wheat can be contaminated with more than one mycotoxin (3, 4, 5). Bedding material, which is typically consumed by birds, can also be contaminated with mycotoxins which adds another source of risk (5). The exposure of birds to mycotoxins can then result in negative toxic effects, with the presence of multiple mycotoxins increasing risk through additive, synergistic, and antagonistic interactions (6,7,8).

THE EFFECTS OF MYCOTOXINS ON THE LAYING BIRD

Consumption of mycotoxins can affect any system in the body and potentially cause varying degrees of lesions and clinical signs. The most common types of mycotoxins that affect poultry through feedstuffs are aflatoxins (AF), ochratoxins (OTA), fumonisins (FUM), type B trichothecenes such as deoxynivalenol (DON), zearalenone (ZEA), and type A trichothecenes which includes T-2/HT2 toxins (9). However, further advances in mycotoxin research indicate that the interactions between mycotoxins and the role of lesser-known mycotoxins have a greater impact on animal systems than previously thought (10). As a result, the impact of mycotoxins on animals can be quite complex.

Birds can be more tolerant to mycotoxins than other species of livestock, but even within avian species, the effects of mycotoxins can vary significantly. For example, turkey breeders may be more susceptible to ZEA when compared to broiler breeders (11). The impact of these toxic metabolites can still be cause economical losses; particularly in the poultry sector, where long term health effects can be carried over the life of the flock and beyond, which would diminish animal welfare (2). Mycotoxins have the capability of modifying DNA, RNA, and hinder protein synthesis, which has the potential to be mutagenic, embryotoxic, teratogenic, and carcinogenic (8, 12). In poultry species, particularly laying birds such as broiler breeders and egg layers, profitability greatly depends on the quality of the egg. However, fertility and hatchability are also measured parameters important for profit. Mycotoxins such as AF, OTA, type A and B trichothecenes, moniliformin, ZEA, and FUM have been found to be toxigenic and exert detrimental effects to varying degrees on of egg

production and quality (10, 13, 14). Egg shell integrity, particularly eggshell thickness, shape, color, texture, and cleanliness can be affected by the presence a single mycotoxin or several of these mycotoxins together. For example, ZEA alone did not significantly affect egg production, egg weights, and feed intake in egg layers. However, in combination with AF, it resulted in even greater losses than those in groups that were already contaminated with AF and were showing decreases in these areas (15). Furthermore, some mycotoxins act directly on pathways that affect quality, while some act indirectly. For example, AFB1 can directly affect eggshell quality by inflicting hepatic damage, affecting liver metabolism and thus causing a decrease in vitamins, minerals and enzymes involved in eggshell formation (10). Indirectly, mycotoxins such as T-2 toxins and other trichothecenes can cause eggshell quality issues through a reduction in feed intake through an increase in mouth lesions (10, 16). Inability of the birds to consume feed or have a decrease in feed consumption would lead to a decrease in available nutrients needed for eggshell formation as well as a decrease in overall egg production (13, 17).

Although eggshell quality may be of higher importance in table egg layers, it should also be considered of importance in broiler breeders as the shell is involved in gas exchange and provides a natural barrier from the outside environment to keep pathogens from translocating into the egg (18). Increased number of cracks and thinner eggshell eggs can harbor harmful pathogens that could be introduced into the incubators and increase the number of exploders, hence contaminating the rest of the batch with bacteria. It is important to note that protective mechanical barriers become extremely important in maintaining exposure of mycotoxin-producing fungi from the litter bedding systems which can contaminate eggs. Hen housing environment, particularly those in litter bedding systems, have a higher risk of fungi exposure and therefore a higher potential for mycotoxin contamination, which increases significantly after the second week of storage (5). Additionally, the presence of mycotoxins can further increase during egg storage particularly if exposed to high humidity and higher temperatures, which is ideal for the growth and translocation of pathogenic organisms such as bacteria and fungi into the egg (5).

In breeders, fertility and hatchability can also be affected by mycotoxin exposure. Fusarium mycotoxins, AF, and diacetoxyscirpenol (DAS) have all shown to decrease hatching eggs (13, 19, 20). Although the presence of mycotoxins does not seem to significantly impact female fertility, male birds do show signs of dose dependent infertility (19, 20). The reason as to why hatchability may be impacted and regardless of adequate fertility, may be due to changes associated in the chemical composition of the egg and not with sperm storage in the hen. However, hatchability may not always be impacted as it was the case in studies where broiler breeders showed an increase in hatchability with low levels of DAS and fusarium mycotoxins in naturally contaminated diets (14, 20). In males, mycotoxins such as DAS have shown to reduce reproductive organ weights and cause degenerative processes of the testis, which leads to reduced spermatozoa quality and an overall decrease in semen quality (20).

Mycotoxins also affect rapidly-dividing cells, particularly those from the gastrointestinal epithelium and immune system (21, 22, 23). Studies have shown shifts in the gastrointestinal microbiota by mycotoxins which can influence the number of dirty eggs and potentially translocate harmful pathogens into the hatchery and the progeny housing (5).

MYCOTOXINS CARRY-OVER EFFECTS OF ON THE PROGENY

Carry-over of toxic metabolites into animal-based food products is something of concern due to the potential harmful effects in humans and domestic animals (24, 25). Due to that mycotoxin compounds can be accumulated into tissue and fat and eventually reach milk and eggs, continuous monitoring has become part of food safety regulation (25). In eggs, the commonly found mycotoxins are ZEA, AF, FUM, OTA, trichothecenes, and moniliform. However, most of the research has been done with AF carry-over into eggs from contaminated feeds.

Research continues to show that mycotoxins affect hatchability to varying degrees despite eggs being fertile (20, 26). A significant component to poor hatchability is due to the embryotoxic and teratogenic effects of mycotoxins in chicken embryos which has been reported with AF, OTA, T-2 toxin, and many others. Although the mechanism of action for early embryonic death is not completely understood, one theory may be related to the thickness of the eggshell, which affects gas exchange and allows for moisture loss during the incubation period (14). Mycotoxins such as FUM are known to affect complex sphingolipids, ceramide, and enzymatic metabolism; particularly those associated the development of embryonic chick brain (8, 12). Early embryonic death lesions observed are associated with hydrocephalus abnormal neck and beak formation (12, 26). Other pathological changes such as tissue enlargement and hemorrhages can be observed in heart, kidneys, muscle, lungs, intestines, and testes. Incomplete closure of the umbilicus has also been reported in similar studies (26, 27).

Mycotoxins are well known for their properties as immunosuppressive agents (21). Immunosuppression is a key concern; particularly in poultry production systems where immune function plays a key essential role in maintaining overall health and welfare while improving performance and preserving profitability (28). The progeny of breeders exposed to mycotoxins through feed has been reported to show a higher incidence of unthrifty and immunosuppressed chicks (19). Breeders exposed to AF and OTA have been reported to have chicks with decreased cellular and humoral responses (29). The effect on the immune system is due to the impact of mycotoxins on rapidly diving cells that are involved in immune-mediated activities and by hindering the communication network of the cellular and humoral systems (21).

HOW TO MANAGE MYCOTOXINS: TESTING AND MITIGATION TECHNIQUES

Prompt recognition of mycotoxin contamination in feeds and feedstuffs has its limitations. On routine testing of samples globally, more than 1 mycotoxin is found in grains routinely used in poultry feeds (2). The most common mycotoxins found were FUM, type B trichothecenes, ZEA, fusaric acid, and other emerging mycotoxins (10). Typically, the number of mycotoxins being tested for at the feed mill level is limited to one to four common types. This would exclude a vast majority of the existing mycotoxin contaminants.

In poultry, mycotoxins are suspected when birds are exhibiting feed refusal which tends to resolve shortly after a change in the suspected affected feed (30). Clinical signs may not be present or if observed, typically associated with morbidity, mortality, and performance losses such as stunted growth, decreased feed conversion and noticeable decrease in egg production. In cases where further testing is performed, mycotoxin feed screening of the suspected feed or feed from the crop of affected birds may be able to provide further evidence of the presence of these compounds. Further testing can also be performed in birds as pathological changes can be suggestive for the presence of mycotoxins; however, these are not always definitive.

Currently, there are several forms of intervention which can be applied to the feed which can mitigate the effects of mycotoxins in the body and from potentially going further down the chain into the eggs and the progeny. Adsorbents, such as those that contain glucomannans extracted from the outer cell wall of yeast, can physically bind some of these toxic chemical metabolites and prevent them from being absorbed from the gastrointestinal tract (31, 32). Dietary additions of adsorbents in the diet have shown to be very practical in animal production systems as well as possible mitigating strategy to reduce the mycotoxin effects associated with performance losses such as feed conversion and egg production (33, 34, 35).

CONCLUSIONS

The interactions that mycotoxins have are not yet completely understood. Whereas sometimes clinical mycotoxicosis is evident, chronic exposure to a combination of mycotoxins may not be clinically obvious. However, the ramifications of contaminated feed and bedding materials used in everyday poultry production systems can be detrimental to achieving optimal performance, improve animal welfare, and maintaining profitability. Mycotoxins continue to be an area of interest, particularly because of carry-over and the potential to enter the food chain. Their impact in laying type birds and their progeny should be taken into consideration when establishing a mycotoxin reduction program. Adsorbents that manage the risk of multiple mycotoxins simultaneously, should be used to mitigate the clinical signs associated with mycotoxins and potentially help maintain good egg production and decrease the risk of carry-over to the progeny.

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COMPARISON OF SAME-HEN YOLK AND SERUM ANTIBODY LEVELS USING THE ELISA TEST FOLLOWING VACCINATION WITH TYPICAL VACCINES USED IN COMMERCIAL LAYING HENS

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INTRODUCTION

Many commercial egg layer quality assurance plans require ranches to maintain flock health programs that include periodic monitoring to evaluate vaccination response and disease exposure. Without the assistance of a veterinarian or other trained person, avian venipuncture may be a difficult feat. It is also time-consuming and may induce added bird stress. Egg yolks have been successfully used in the past to evaluate flocks for MG exposure (1). Our study compares serum and yolk samples from individual laying hens in order to see if egg yolk antibody level is sufficiently correlated with serum antibodies to be useful in surveillance programs. In this study, the ELISA test is used to compare measurable antibody response in egg yolk vs. serum to some typical layer vaccines, i.e., Newcastle disease (ND), infectious bronchitis (IB), avian encephalomyelitis (AE), and *Mycoplasma gallisepticum* (MG).

MATERIALS AND METHODS

Samples were obtained from one hundred twenty (120) White Leghorn laying hens housed at Shepherd Poultry Farms LLC in Spanish Fork, Utah. The hens were numbered and tagged and remained in the same location in the flock to prevent an increase in stress levels. On sampling dates, hens were removed from their cages and placed into individual transport cages so that each egg could be matched to the corresponding hen's blood sample. A minimum of 0.5 mL of blood was obtained from the brachial (medial wing) vein and placed in a 3 mL microcentrifuge tube. Tubes were labeled with the hen's identification number. Eggs were collected during the same time and labeled with the same identification number. Upon arrival at the lab, the whole blood was spun at 6,500 rpm for five minutes at 28°C, after which serum was immediately drawn off and placed into clean, labeled microcentrifuge tubes. Eggs were cracked and internal contents allowed to gently fall into a sterile petri dish so as not to rupture the yolk. An 18 ga needle and 3 mL syringe was inserted into the whole yolk, and 1 mL of yolk was drawn out. One-half (0.5) mL of the yolk was then placed into a 3 mL microcentrifuge tube pre-filled with 0.5 mL of a 0.85% saline solution. Tubes were labeled with the yolk's hen number. All microcentrifuge tubes, serum, and yolk were placed sequentially (e.g., serum 1, yolk 1, serum 2, yolk 2, etc.) in two 96-well microcentrifuge tube racks. Fifty to100 µL of both serum and yolk were placed in the same sequential order in two template plates. Yolk samples were vortexed at 3,200 rpm for three to five seconds, or until yolk and saline were thoroughly mixed before being placed into template plate well. Three hundred (300) µL of Zoetis dilution buffer were dispensed into each well of two additional template plates. Subsequently, 6 µL of sample were placed into the dilution plate wells, matching the order of the previous template. Both dilution plates were covered with Parafilm (Bemis Company, Inc) and placed on a plate rotator for 15 minutes at 100 rpm. Remaining testing procedures followed Zoetis ELISA instructions (2). Samples were analyzed using Bio-Tek Instruments, Inc. ELx800 Universal Microplate Reader (Filters 405, 450, 490, and 630) and Zoetis ProFILE3 Animal Diagnostic System software.

RESULTS

Sample-to-positive ratios were analyzed using the Pearson's correlation coefficient method, Student's T-test, and a beta hypothesis test. See Table 1 for results. P-values of $p<0.01$ are considered significant and a Pearson's correlation coefficient of $r > 0.70$ is considered to be a strong correlation between variables. The p-values in this test indicate that the null hypothesis may fail to be accepted and the r-values indicate that each test has a strong correlation. β-values indicate that in all four tests, the chance of a Type II error is 0.0%, demonstrating that the data from

each test may be considered "significantly significant."

DISCUSSION

During the evolution of this project, three different methods of yolk sample preparation were tried. One method involved larger yolk sample sizes: 5 mL of egg yolk were added to 5 mL of a 0.85% saline solution. This method of sample preparation proved to be extremely accurate; however, it was inefficient, expensive, and took two days to run the test. Another method used no saline: egg yolk samples were placed directly into the dilution plate. Because of the viscosity of the yolk, inconsistencies in the amounts of yolk placed into each well occurred, and although some of the yolk samples matched the serum samples, the majority had no significant correlation. Results from the third and chosen method (described in the Materials and Methods) proved to be the optimal procedure for sample preparation. Less supplies were needed, it was relatively time-efficient, and results had a significant correlation.

As noted in the results, there was a significant correlation between the serum S/P ratios and yolk S/P ratios. The p-values indicated no significant difference between serum S/P ratios and yolk S/P ratios, and the β -values were minimal, allowing for the failure to accept the null hypothesis.

Under circumstances where producers or veterinarians are unable to obtain blood from laying hens, the procedure outlined in this paper has shown to be useful for a producer, veterinarian, or laboratory technician to utilize egg yolks for flock surveillance testing for Newcastle disease, infectious bronchitis, avian encephalomyelitis, and *Mycoplasma gallisepticum* antibodies. Not only would it be beneficial in emergency situations where a veterinarian trained in avian venipuncture is not available, but simply, it is much easier to collect eggs than to handle, restrain, and draw blood from a hen. Additionally, it does not provide the same needle-stick hazards as venipuncture, and it would significantly decrease the amount of added stress imposed upon the hens. This technique, however, may increase laboratory time in preparing samples.

Further testing where both eggs and blood are collected randomly throughout the flock would indicate the significance of mass testing and random sampling when using egg yolks rather than exclusively same-hen serum to yolk sampling.

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(A detailed version is anticipated to be submitted to a peer-reviewed journal.)

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Table 1. Pearson correlation coefficient (r), T-test p-value (p), and beta test (β) values for Newcastle disease (ND), infectious bronchitis (IB), avian encephalomyelitis (AE), and *Mycoplasma gallisepticum* (MG) antibodies levels comparing same-hen serum and egg yolk samples.

	r	p	β
ND	0.81	<0.001	0.0%
IB	0.78	<0.001	0.0%
AE	0.86	<0.001	0.0%
MG	0.84	<0.001	0.0%

Figure 1a. ELISA sample-to-positive ratio of same-hen serum and yolk sample: Newcastle disease.

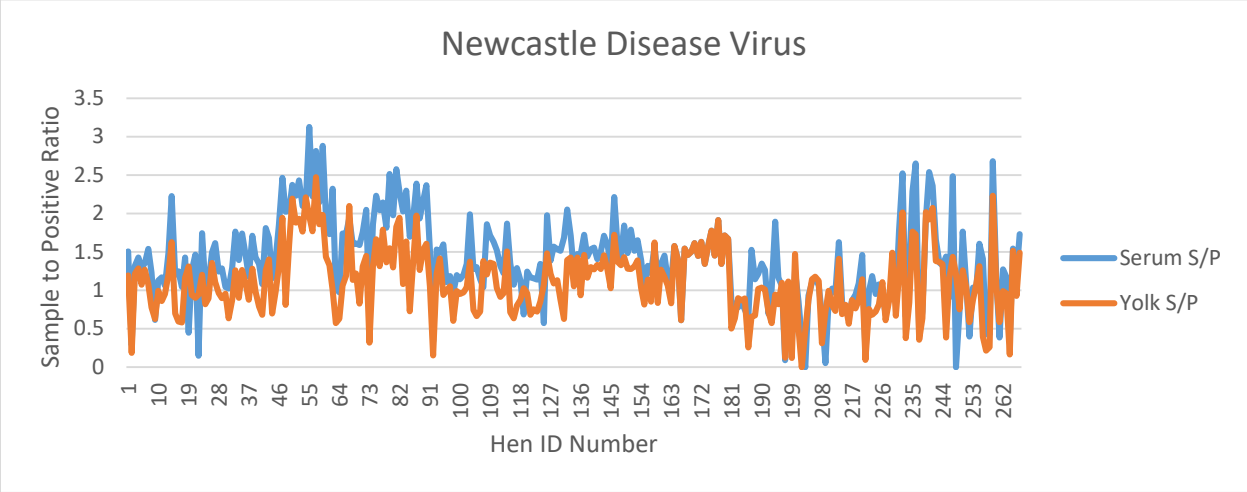


Figure 1b. ELISA sample-to-positive ratio of same-hen serum and yolk sample: infectious bronchitis.

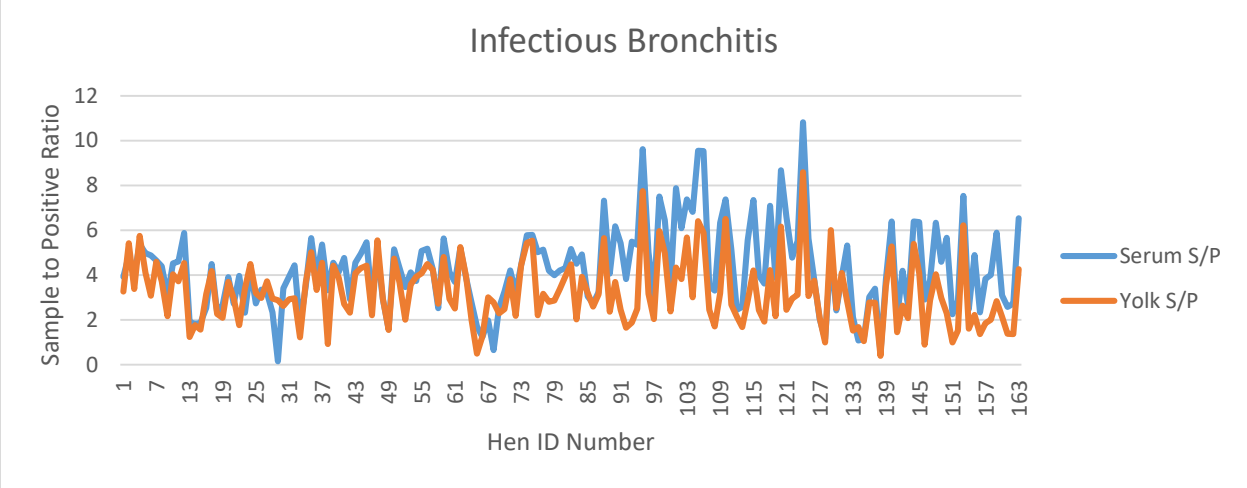


Figure 1c. ELISA sample-to-positive ratio of same-hen serum and yolk sample: avian encephalomyelitis.

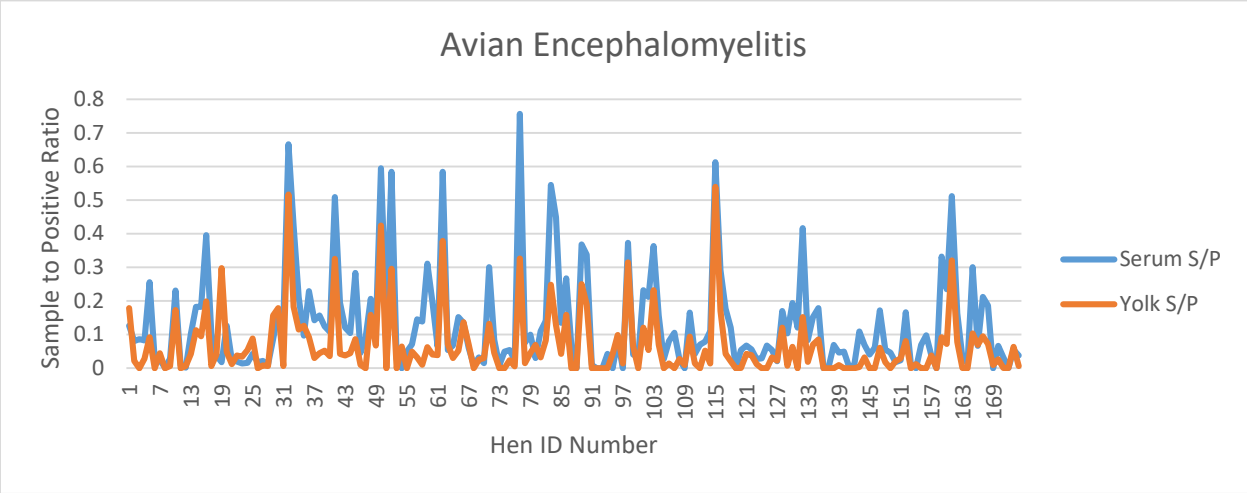
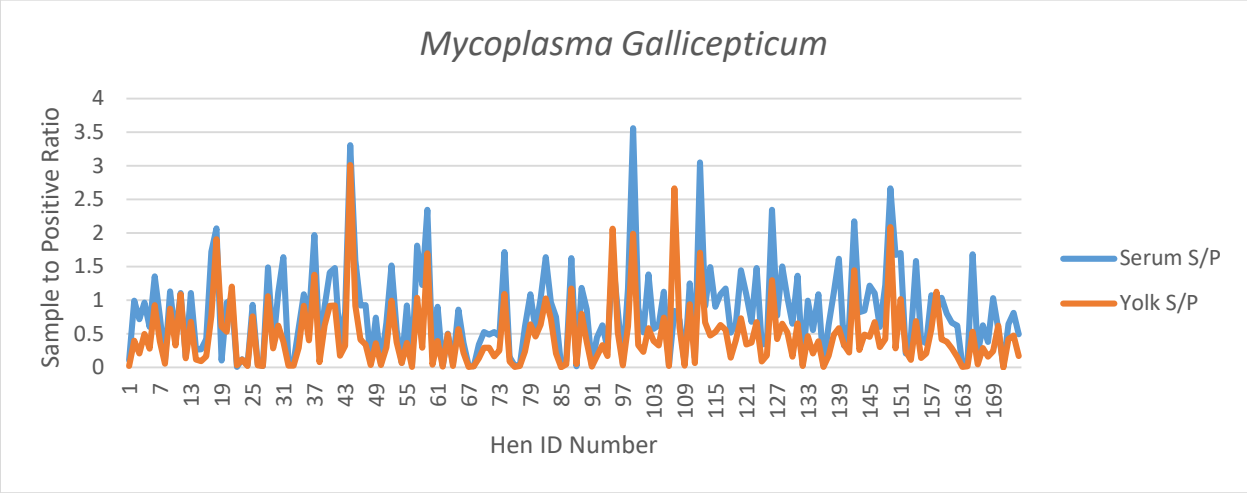


Figure 1d. ELISA sample-to-positive ratio of same-hen serum and yolk sample: *Mycoplasma gallisepticum*.



OUTBREAKS OF ASPERGILLOSIS IN YOUNG TURKEY POULTS PROBABLY DUE TO HATCHERY CONTAMINATION

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SUMMARY

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

A retrospective study performed by searching the CAHFS electronic database on the cases diagnosed with aspergillosis in turkeys submitted to the Turlock and Tulare laboratories for the years 2018 and 2020 identified 135 cases. An assumption was made that the turkey poults submitted between the ages day-old and seven days and diagnosed with aspergillosis were probably due to the hatchery contamination. Sixty-five out of 135 such cases were identified. Except for mild respiratory signs and occasional neurological signs and increased mortality, there were no other clinical signs. These poults had a few small pale yellow foci ranging in size from 1-2 mm to 3-4 mm in the air sacs and lungs. Microscopically, the lesions in one to seven-day-old poults were composed of giant cell granulomas associated with mycelia of *Aspergillus* spp. *A. fumigatus* was most commonly isolated from the lesions.

Older poults between eight and 28 days (45/135 cases) of age had a few to numerous larger pale yellow nodules throughout the lungs and air sacs and occasionally in the brain. Most of these latter cases most likely were originally diagnosed in poults with aspergillosis when they were between one and seven days old.

HOW THE BROILER INDUSTRY WILL BE AFFECTED BY THE CURRENT EVENTS

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"CURRENT EVENTS" AFFECTING THE MEAT BIRD INDUSTRY

There have clearly been a lot of "current events" to consider in the last several years that could/should be considered significant to the meat bird industry. I'm not sure that it is any more than any other 5-year segment of time in the past 50 years, but it sure seems like it. Three "events" that feel most significant, for me, are:

1. Labor/Technology
2. Information and communication
3. One Health

THREE THINGS ABOUT THE THREE THINGS

Labor/Technology

Introduction. We are a labor-intensive industry. Every harvest plant (of significant size) probably has between 500 and 700 employees per shift to do all that needs to be done to get chicken into the appropriate package for sale. This might be twice the number of people for the same harvest numbers in other parts of the world (like Europe). Keeping up with the training and oversight of this many people is a challenge. During COVID, employees had many challenges in their own lives including child care, parent care, community needs, etc. So, the dependability of workforce from one day to the next became a little unpredictable. But…. the number chickens coming to the harvest facility was much more dependable…they are coming.

The technology available to the poultry industry has fortunately grown with our needs. What you can do with a scale, meter, blood sample, fecal sample, bird rinse, muscle biopsy today is thankfully (or overwhelmingly) different and evolving.

1. Automation in Processing. It is inevitable that more things are, and will continue to be, automated. One of the most labor-intensive areas of a harvest facility is the deboning department. Both front half (breast) and back half (legs and thighs) are candidates for automation. If we are heading for more auto deboning, the process will demand a stronger focus on flock/bird uniformity. The more uniform the bird presented to an auto deboning machine, the better job it can do at cleanly (and safely) removing the meat. We don't often describe the uniformity of a farm/house in the meat bird industry but we probably should. It is done routinely when raising parents but not common in meat bird management. Uniformity can be addressed through sizing equipment at processing and perhaps that will be successful enough – for a while. The auto deboning systems may also continue to push the live side back to gender separation. I see no down side to understanding and measuring uniformity – no matter what the processing technology holds.

2. Bacterial Fingerprinting. The problem is not just viral or bacterial. It is not just genus and species. It is now basically whole genome sequenced and has basically a "fingerprint." Of course, this means the bacteria that are yours, are YOURS. Or the regionality of some bacteria are (or can be) well characterized.

The opportunity to do great epidemiology has really exploded. Poor epidemiology (not enough data, poorly analyzed) can also be a component of all this information. To really understand some of the changes from one year to the next, or one complex the next, one product vs another, one breed vs another, one management style vs another has taken a big step.

It seems we may be more scared of this technology than excited by it. If used right, we will be able to make some very good (and helpful) decisions. Once we get past the explosion of information overload.

3. Vaccine technology. Vaccine technology has changed so much in the last five years, and it needed to. It is not debatable that modified live vaccine has been a huge tool to the commercial poultry industry in the past 50 years. When farms were small and spread out, growth rates were slow, clinical disease was prevalent, a modified-live vaccine was a fantastic step forward. They worked relatively well and they were "inexpensive." We (the industry) could even make them less expensive by "cutting the dose." Bigger farms that are closer together, a day of lost growth rate, and the recognition that the mild disease caused by many MLV vaccines can have implications to significant loss of feed conversion or antibiotic use, has led to a significant move towards more expensive vectored type vaccines.

Newcastle, infectious bursal disease, and laryngotracheitis can all be addressed using the appropriate Marek's or fowl pox vectored products. More, better, quicker approaches to vaccination without causing a mild form of the disease clearly have a bright future.

Virtually every meat bird company utilizes (and struggles to feel good about it) autogenous vaccines. There are all inds of rules associated with getting them made (in the name of safety) but little or nothing done to assure (or measure) efficacy. I am not suggesting this is the governments job as much as a thing that we have figure out. Faster and better approaches to catching up to the evolving bacteria and viruses (made clearer through the previously discussed technology) will be available. You can see this innovation coming fast due to the post-COVID technology boost.

Information and Communication

Introduction. As an industry, this has not been our strength. Because we have chosen to not be proactive, we generally end up being reactive. We often think we know better than others what is right and wrong about what we do, so we can come off a little egotistical or condescending. We often suggest that many of the things people are worried about are silly. We need to rethink that.

1. Methods of communicating to us. Consumers and customers want and demand a relationship with the companies providing them with food. It is harder and harder for the industry to use an allied industry group as the single source of credible information. Because, in many things, general agreement among production companies on the appropriate answer to a question is getting harder to find. A second, perhaps even more significant trend, is that the consumer/customer wants a personal relationship with an opportunity to hear from the company in as "normal" talk as possible. Questions and comments can come into a company in so many ways now compared to five years ago. Letters (still), fax (still), chat, phone, social media, text, and web forms are all often utilized to give a company feedback (depending on the company). If a consumer wonders it, they can easily ask it. And…. they expect (and deserve) a personal response. The pace that an issue can proceed (good or bad) is hours not days. People that buy your products deserve an opportunity to ask some questions about the food they will be serving to their family. Really good companies get virtually the whole company involved in addressing questions and concerns from their consumer/customer.

2. Techniques used to communicate back. Words are important. Simpler is better. Seeing is even better than really well written words. Showing video clips or even real-time camera footage can be powerful and appreciated. It takes time and expertise to get good at showing people how something works or what a situation looks like.

What you aspire towards is at least a part of almost every important message. Many scientists have spent years and years learning big complicated words to discuss complex subjects. They then need to spend just as much time learning to explain the same thing without those words and complexity. The industry is beginning to show some better skills in communication and relationship building – but it takes time and effort AND is best done because you want to not because you must.

3. Label claims and certifications. Some very important aspects of product differentiation (and things that are deemed important to the consumer) often come down to "3 words" on a label "claim." This often seems impossible. So, often, the full explanation of what the "3 words" really mean sits on a website somewhere in several paragraphs. Although the industry can debate the importance of the various claims, what we should all want is a commonly accepted and defended definition (set of rules). A label claim with multiple definitions is not good for anyone, particularly the consumer.

I used to believe in the value of certifications more than I do now. They are generally very complicated and difficult for consumers to relate to. There are multiple programs associated with the same subject (welfare, food safety, etc.), many with slight differences (or not so slight). Certification plans would like to be considered the "gold standard" for an area but these subjects are often contentious and highly debated. There is even some confusion surrounding the difference between the audit instrument and the auditor. If the rules (audit instrument) are written clearly the auditor is just validating compliance with little or no opinion or bias. Ultimately the value to the consumer comes from their "understanding" of the criteria and how (if) it fits with their concern (or desire). Even with all the skepticism of consumer value, there can be considerable positives within a chicken company. A certification program can force a company to define the things they believe are important in areas such as animal warfare, food safety, allergen control, feed ingredients, etc. Clear criteria and an impending audit from a third party can help get (or keep) your company focused on doing what you said you were going to do. Again…simple and clear is best.

One Health

Introduction. The state of health of people, animals, and the environment are all related. This is hard to argue but there will be quite a bit of discussion and debate around what decisions are embracing this concept.

1. Feed ingredients, feed efficiency, and growth rate. What's in the feed you feed to your chickens. The meat bird industry has done some amazing things the past 40 years in the name of efficiency. Through genetics, nutrition, nutritional supplements, management, and approaches to health management. We need to catch the consumer up on the things we've done and get their feedback. It is likely the balance between efficiency and some of its tradeoffs will need to be re-evaluated.

2. Density. One of those areas that we should (or will be asked to) look at closely in animals (and humans) is appropriate density. When does an area have too many animals? In my opinion we have some areas in the world with too many chickens per square mile (or kilometer). Our answer to this, as we grew, was to expand and enforce better biosecurity. Biosecurity (and common disease containment practices) are only so good. Unless they become very extreme (SPF). I don't believe we can (or should) raise all our meat birds in an SPF housing type setup.

When is a chicken house too crowded? The industry has answered this by measuring and judging our ability to maintain health and efficiency while adjusting the number of chickens within a given management scheme. The question that needs to be addressed going forward will be around space appropriate for chickens to allow them to exhibit normal behaviors. Is it important? What behaviors? How do you measure it or judge it?

Density and lay out time are both very powerful tools that are constantly being discussed. It is likely with some of the evolving approaches to feed and management, whatever you thought was "right" before should be reconsidered.

3. Viral mutations and bacterial plasmid "trading." New things created in a poultry environment is likely a "heads up" we should be paying attention to. Vaccines or vaccine application technique that encourage viruses to change is not likely a sustainable future. No matter how fast we get at making new vaccines. Plasmid movements from one bacteria to another has likely gone on for a long time, but now that we can "see" it we will need to pay attention to it.

START, STOP, CONTINUE BASED ON "CURRENT EVENTS"

1. Start knowing what your birds weigh at different ages (with some opportunity to measure uniformity).
2. Start acknowledging that the whole thing is a journey and we are a slow-moving ship making changes that take time.
3. Stop using biosecurity as an excuse for why you won't show people what the inside of a chicken looks like.
4. Stop thinking that chickens are just chicken.
5. Stop being worried about saying we do some things wrong and could do them better.
6. Stop thinking that every exciting technical advance needs to go "into" the chicken (feed, water, injection).
7. Continue pushing for clarity and consistency in label claims.
8. Continue treating consumers like your business depends on it (because it does).

INTRAMUSCULAR CPG-ODN PRIMING AUGMENTS OXIDATIVE BURST, DEGRANULATION, AND PHAGOCYTIC POTENTIAL OF IMMUNE CELLS IN CHICKENS

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SUMMARY

The unmethylated cytosine phosphodiester guanine-oligodeoxynucleotides (CpG-ODNs) activate chicken Toll-like receptor-21 (TLR-21) have immunoprotective roles against several bacterial pathogens. Our recent studies demonstrated that the antimicrobial function of CpG-ODN in chickens is mediated through the enrichment of various immune cell compartments. In this study, we assessed the effects of intramuscular CpG-ODN on phagocytosis, degranulation, and oxidative burst functions of heterophils and monocytes 48 hours post-CpG-ODN administration. We also developed flow cytometry methods for the evaluation of oxidative burst, phagocytosis, and degranulation. Our study revealed that CPG-ODN priming enhances phagocytic immune cells' oxidative burst, degranulation, and phagocytic capacities in chickens. We observed significantly increased oxidative burst activity ($p=0.0303$) in CpG-ODN treated group (54%) than the control group (32%). The phagocytosis percentage of CpG-ODN treated chickens (38.34%) was higher than saline (23.03%) in the monocyte and heterophils population. The degranulation assay revealed that CpG-ODN stimulated cells had higher activity (35-38%) than the non-stimulated group (12-14%). Our study results suggest that CpG-ODN modulates multiple facets of the immune system to induce antimicrobial immunity in chickens, which has excellent potential as an alternative to antibiotics in chickens.

INTRODUCTION

In the commercial chicken industry, bacterial and viral diseases cause significant economic losses due to increased mortality and decreased production. Usage of antimicrobials as a prophylactic and growth-promoting agent has raised public health concerns due to antimicrobial resistance development (7). Antimicrobial resistance is a global problem and one health issue. Therefore, the poultry industry is urgently looking for alternatives to antibiotics (1, 2). CpG-ODN administration through parenteral routes such as intramuscular (IM), subcutaneous (SC), *in ovo*, intraperitoneal (IP), and intrapulmonary (IPL) routes has shown significant immunoprotective effects in chickens (3, 4, 5, 6, 8). We have just started the understanding of the CpG ODN-induced antimicrobial mechanisms. A greater understanding of CpG-ODN-induced immune-stimulatory mechanisms and identifying the cells involved in this mechanism are essential to establish the potential use of CpG-ODN as an alternative to antibiotics in the chicken industry.

MATERIALS AND METHODS

Synthetic CpG-ODN (TCGTCGTTGTCGTTTTGTCGTT) free of endotoxin and produced with a phosphorothioate backbone was used in our study (Operon Biotechnologies Inc., Huntsville, AL). Synthetic CpG-ODN was diluted in sterile, non-pyrogenic saline. CpG-ODN (50 µg/bird) and saline (control group) were injected intramuscularly (IM) into two to three-week-old broiler chickens (n=5 birds/assay/group). Blood was collected into heparinized tubes from both CpG-ODN and saline groups after 48 hrs of CpG-ODN administration to analyze oxidative burst, phagocytosis, and degranulation in monocyte/macrophages and heterophils. Phagoburst® commercial kit was used to detect oxidative burst with some modifications. Briefly, phorbol 12-myristate 13-acetate (PMA), a PKC activator, was added to stimulate immune cells and incubated at 37°C only for 5 minutes. This step was followed by oxidation of dihydrorhodamine (DHR) 123 into Rhodamine 123, a fluorescent-labeled substrate. Phagocytosis was quantified using Phagotest® kit with some modification to the protocol. Fluorescent (FITC) labeled opsonized *E. coli* was added into blood samples to stimulate phagocytosis. Both experiments were included with RBC lysis and leukocyte staining steps. For degranulation, primary CD106a and AF488-labeled secondary antibody were used, and data

acquired using flow cytometry and analyzed by FlowJo software.

RESULTS AND DISCUSSION

Quantitative functional analysis revealed significantly higher (p=0.0303) oxidative burst activity in CpG-ODN treated group (54%) compared to the saline given group (32%). Heterophils are considered functional homologs to mammalian neutrophils but have less oxidative burst killing capacity as they lack myeloperoxidase, which is crucial for this function. In this study, our results revealed that CpG-ODN can increase oxidative burst in chicken heterophils. Phagocytosis was also higher in CpG-ODN treated chickens (38.34%) than saline control (23.03%). The phagocytic activity was significantly higher (p=0.0043) in the monocyte/macrophages and heterophils population. The degranulation in the CpG-ODN group was higher (35-38%) than the saline control group (12-14%). The assays developed in this study will help study the immune cell functions during host-pathogen interactions and vaccine development.

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IBV STRAINS ASSOCIATED WITH FALSE LAYER SYNDROME: SURVEILLANCE IN LAYER FARMS AND IBV GENOMIC STUDIES

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SUMMARY

Infectious bronchitis virus (IBV) is a coronavirus that mainly induces a respiratory disease but has also been associated with false layer syndrome (FLS). FLS is a condition that occurs in laying hens after being infected with distinct IBV genotypes during their first week of life. The reproductive tract tropism of certain IBV genotypes infecting chicks results in oviduct atrophy and false layers after sexual maturity. The use of IBV vaccines at day of age alleviates the clinical outcomes of this infection. However, the consequences of vaccination in the wild-type IBV field variants are not clearly understood. In the present study, we have performed a molecular surveillance in a commercial layer farm with a history of FLS in order to understand the relationship between the live-attenuated vaccines used in chicks and pullets and the wild-type strains circulating in the environment. We are currently working on comparing full genome sequences of IBV viruses found in databases to genomic sequences obtained from FLS-associated IBV strains.

INTRODUCTION

False layer syndrome (FLS) is a disorder caused by infections with specific infectious bronchitis virus (IBV) strains at an early age, leading to early oviduct damage that results in reproductive failure when the hens reach sexual maturity. In addition to the ordinary IBV-induced respiratory disease in laying hens, FLS causes several reproductive problems such as drops in egg production, poor hatchability, hindered development, and cystic distention of the oviduct (1). Only few IBV strains have been associated with FLS in chickens, such as QX and QX-like strains in Asia and Europe and DMV/1639 in the US and Canada (2, 3, 4, 5).

According to De Wit *et al.* (2011), one efficient strategy to prevent FLS is the use of live heterologous vaccines (Mass strains) at day of age, a second dose at 14 days (Mass + Conn) and another boost at 25 days including three IBV serotypes (Mass + Conn + Ark) (6). However, the substantial usage of live-attenuated vaccines might allow the interaction between wild-type and vaccine strains, leading to the generation of novel IBV variants that can eventually circumvent the immunity induced by such vaccines (7). Studying the IBV dynamics in layer farms is of particular interest since these birds are heavily vaccinated at an early stage of their lives and receive inactivated IBV vaccines before the lay onset.

This study aims to understand the relationship between the live-attenuated vaccines used in layer chicks and pullets and the IBV strains detected from tracheal samples of these vaccinated birds. In addition, we investigated the IBV strains circulating within hens in lay (30 and 36 weeks of age) by testing their tracheas, kidneys, and cecal tonsils. This molecular surveillance was performed in a commercial layer farm that had a history of false layer disorder. We are currently working on comparing full genome sequences of IBV viruses found in databases to genomic sequences obtained from FLS-associated IBV strains.

MATERIALS AND METHODS

Sample collection. Samples were collected from a commercial layer farm with a history of clinical FLS. Tracheal samples were collected from 120 birds at 1, 7, 14, and 21 days of age (30 samples each). Tracheas, kidneys, and cecal tonsils were collected from laying hens at 30 and 36 weeks of age. All samples were collected for molecular surveillance using an IBV RT-PCR assay.

Flock history. Chicks were vaccinated at the hatchery with an IBV Ma5 vaccine. At 15 days, they received a vaccine containing IBV Mass and Conn types. At 25 days of age, they received Mass, Conn and Ark strains. Finally, at eight weeks, chicks received a boost vaccine containing Ark and H120 IBV types. All vaccines used were live-attenuated.

RNA extraction and RT-PCR. Viral RNA extraction from tissues was performed using the RNeasy Mini Kit (QIAgen, Valencia, CA). The RT-PCR was performed using the QIAgen One Step RT-PCR Kit (QIAgen, Valencia, CA. Primers S17F and S18R were used to amplify an 817-bp fragment containing the hypervariable region of the S1 gene of

IBV. The phylogenic trees were made using Geneious Prime 2020.1.1.

RESULTS AND DISCUSSION

The molecular surveillance from chicks' tracheas at one day of age resulted in one sequence similar to Ma5 vaccine (Figure 1A). At seven days of age, most of the sequences (n=20) were also similar to Ma5. These results reflect the vaccination strategy used at the hatchery with an Ma5 vaccine. Interestingly, two sequences obtained from seven-day-old chicks showed high similarity to GA04 (Figure 1A). These two IBV strains might be representing the variability resulted from the mixing of the wild-type viruses and vaccine strains in the field.

At 14 days, 17 IBV sequences were obtained. All of the sequences presented with high homology to Mass strains, which is compatible with the vaccination used at day of age (Figure 1B).

At 21 days, 32 samples were positive to IBV and showed high homology to GA04 (Fig. 1C). In addition, three samples showed high homology to PA 171/99, Canada 18, and DMV/1639. These three sequences demonstrate that the wild-type IBV variants that are likely causing FLS are still present in the field despite the heavy live-vaccination strategy used in this farm.

Only seven samples collected from laying hens were positive for IBV: five cecal tonsils, one trachea, and one kidney. From the five cecal tonsil samples, two were genotyped as GA04, two as GA98, and one as DMV/1639 (Fig. 2). The kidney sample had an IBV with high homology to GA04 (Figure 2). Lastly, the trachea sample had an IBV similar to PA/1220/98. The homologies for these results are presented in Table 1.

The results from this molecular surveillance study demonstrate the complexity of a field scenario in terms of IBV challenge in laying hens that are heavily vaccinated against IBV with live-attenuated vaccines. Several IBV genotypes are mixing in the environment, creating an opportunity for viral evolution and potentially for the emergence of novel IBV genotypes that evade the immunity elicited by vaccines. Furthermore, these results reinforce the need of molecular surveillance and encourage our upcoming studies on the whole-genome sequencing of IBV strains associated with FLS.

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Table 1. Homology matrix of infectious bronchitis virus sequences from 30- and 36-week-old laying hens with a history of false layer syndrome.

Sample	Genotype	Query coverage (%)	Homology (%)
30-week-old cecal tonsil (3)	GA04	100	87.5
30-week-old cecal tonsil (4)	GA04	100	87.2
30-week-old kidney (5)	GA04	100	85.7
36-week-old cecal tonsil (2)	DMV/1639	92	89.2
36-week-old cecal tonsil (3)	GA98	99	93.05
36-week-old cecal tonsil (4)	GA98	99	93.05
36-week-old trachea (1)	PA1220/98	91	90.5

Figure 1. Phylogenetic analyses of infectious bronchitis virus strains detected in one- and 7-day-old (1A), 14-day-old (1B) and 21-day-old (1C) chickens from a farm with a history of false layer syndrome in Arizona. The phylogeny was performed using an 817-bp fragment of the hypervariable region of the IBV S1 gene. The phylogenetic tree was built using the Genious Prime 2020 2.2.

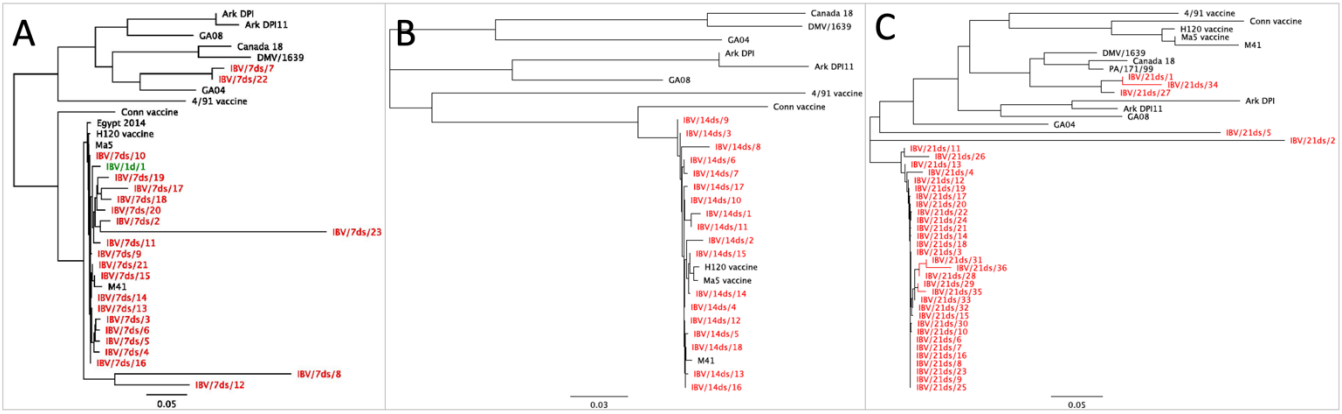
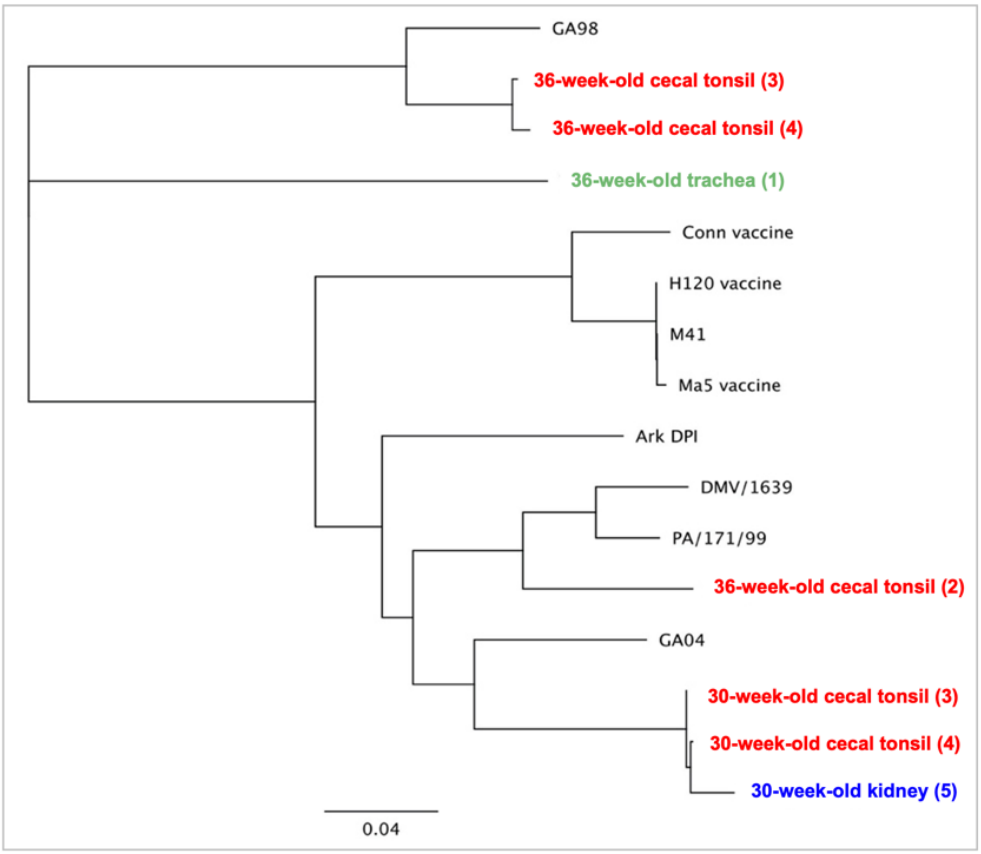


Figure 2. Phylogenic analysis of the infectious bronchitis virus strains detected in 30- and 36-week-old hens presenting with false layer syndrome in a commercial layer farm in Arizona. The phylogeny was performed using an 817-bp fragment of the hypervariable region of the IBV S1 gene. The phylogenetic tree was built using the Genious Prime 2020 2.2.



IMPACT OF COVID ON THE TURKEY INDUSTRY

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SUMMARY

Being in the turkey industry for over 30 years, I thought I had either experienced or knew of just about every possible type of crisis. This included the impacts of high feed prices, vitamin shortages, undercover animal terrorists, barn fires and barn collapses, tornados, floods, highly pathogenic avian influenza, mycoplasmas and salmonella serotypes requiring flock destruction, trucking strikes preventing feed deliveries, live haul and poult truck rollovers, and employee and co-worker accidents. With each crisis companies and the industry have shared "lessons learned" to help others prevent or minimize the impact of a similar crisis and in short – be better prepared for the next one to occur. The H1N1 pandemic of 2009 produced some very useful guidelines on how to ensure business continuity during a pandemic, but this fell far short of preparing us for the far-reaching impact of the COVID-19 pandemic. This paper will discuss the direct impacts of COVID-19 on the turkey industry, the resulting indirect collateral damage and the many ripple effects throughout the production chain.

By far, the most impactful fallout of the pandemic on the turkey industry was the temporary closure and/or slowdown of processing plants and lack of personnel within all segments of the chain. One reference estimated that it decreased turkey processing by 8.3% nationwide (2). In the past, because of a crisis such as a processing plant fire, companies worked together to process flocks ensuring both bird welfare and security of the food supply. During 2020, this collaboration was not possible since all companies were dealing with similar pandemic challenges and closures of plants were regularly occurring to safeguard human health for COVID-19 (3).

A major consequence of plant shut downs or slowdowns were turkey flocks having to be depopulated instead of processed. These decisions were not taken lightly and occurred only when every other possibility was exhausted and flock welfare became compromised. The impact of the COVID-19 global situation also highlighted how interconnected many world resources are and how "simple" things like sufficient availability of carbon dioxide (CO_2) for gas depopulation became an obstacle. CO_2 is a major byproduct of ethanol production. Ethanol plants began to slow or shut down, as the demand for ethanol decreased due to stay-at-home orders and restricted travel nationwide, in addition to labor shortages in their plants due to COVID. As a trickle-down consequence, the volume of readily available CO_2 to facilitate timely and efficient depopulation of flocks was not a viable option in some areas (8).

The lack of plant capacity caused some companies to stop placement of commercial poults for 6 weeks, anticipating that the plant may not be able to handle the normal volume of incoming flocks 20 weeks later. Periods of additional downtime are usually welcomed to upgrade or refurbish facilities. However, with labor, funds and/or some materials in short supply, this opportunity could not be captured. In some cases, there was the cost of having to pay for poults previously ordered from outside suppliers, as they had already invested in the breeders in production.

The impact on the processing plant also included additional resources needed to quickly implement processes recommended by the U.S. Center for Disease Control (CDC) and U.S. Occupational Safety and Health Administration (OSHA) issued to deter the spread of COVID-19 within processing facilities (3). Solutions to structural and operational challenges that some facilities adopted included adjusting start and stop times of shifts and breaks to increase physical distance between workers. Outdoor break areas were added at some facilities to decrease contact between workers. Some facilities installed physical (e.g., plexiglass) barriers between workers; however, this was not practical for all worker functions. Symptom and temperature screening of workers was newly instituted in some facilities and improved in others. Educational materials and communications needed to be developed and provided in multiple languages, which is an onerous task in a plant where over 40 different languages and dialects are spoken. Despite the implementation of numerous precautionary measures within processing plants, positive cases continued to occur. This gave credence to the external factors in play including many workers living in crowded, multigenerational settings and sometimes sharing transportation to and from work, contributing to the increased risk for transmission of COVID-19 outside of the facility itself (4).

Research is necessary to understand why SARS-CoV-2 is such a problem in meat and poultry processing environments and how we can practically

mitigate the problem. A team of Kansas State University researchers recently received a $1 million grant from the US Department of Agriculture – and an additional grant from the State of Kansas – to identify, develop, validate, and deliver practical cleaning and disinfection strategies, plus develop mathematical models to predict and reduce the risk of SARS-CoV-2 exposure in meat and poultry processing facilities.

We were already struggling within the turkey industry before COVID-19 to find and retain employees in all areas from breeder farms to trucking, with low unemployment rates or agriculture just not being attractive to workers (dirty, dangerous, demeaning) cited as the main reasons. With COVID-19 we lost employees short and long term, because of viral infection, viral exposure, or the need to stay home to care for children as schools and daycare centers closed. In addition to being short of personnel, those who were able to continue coming in to work experienced high prolonged levels of stress, fear, anger, frustration, isolation, and fatigue. This occurred at every level as people coped with the lock down, working from home, home schooling, conflicting information from media sources, family and friends who were ill or died, and many difficult personal decisions e.g., is going to the grocery store too risky, how do I ensure my elderly parents are taken care of and is it safe to send my child back to daycare or school. When people are stressed it is difficult for them to focus 100% on their jobs or to absorb changes to routine procedures, consequently training and communication needed to be constantly reinforced.

Due to insufficient labor, or labor that was constantly in flux, the following happened:

- The focus shifted to ensuring that critical activities such as flocks having feed and water, hens inseminated, eggs collected and sanitized, flocks vaccinated, eggs set, and poults delivered occur. However, there were no guarantees that these activities were done well, done on time or as frequently as they should have been, as what could actually be accomplished changed on a daily basis reflecting availability.
- Tasks such as cleaning and disinfection were often short changed and what got accomplished again was dependent on the number of people and time available.
- Routine quality control and testing was either suspended, delayed, or minimized. Personnel who were doing these jobs were often moved into performing more critical tasks, if cross training and competency allowed. Laboratories also had worker shortages at times and only testing required for export or NPIP certification was possible. This certainly varied state by state and throughout the year.
- Elements within standard biosecurity programs such as barn entry and exit procedures, keeping vegetation cut around barns, and pest control not being done to the same level as pre-COVID. With visits by farm supervisors and biosecurity audits limited these nonconformances often occurred for a prolonged time leading to disease challenges on farm. As we all know, there is nothing like a virus to illustrate clearly where all the gaps are in your biosecurity program. Although COVID is not a poultry virus it did show us where unexpected weaknesses were as illustrated in this example.

A turkey company with well documented and audited biosecurity programs in place. Breeder farm health issues minor. Because rodent control was often at the bottom of their farm mangers to-do-list it was outsourced to a reputable pest control service and this had worked effectively for many years. Good service was provided and rodents and other pests were under control. One less thing for the veterinarian and farm manager to worry about. Then COVID happened. This resulted in on-farm labor shortages so biosecurity measures mentioned previously plus composter management were not done effectively. In addition, the pest control company personnel were also impacted by COVID and were not able to provide service to the same level or frequency. These factors plus the extended length of time over which these circumstances occurred combined and this perfect storm led to a major cholera break on multiple lay farms as well as some breeder replacements. Initial mortality escalated, which increased the problem of getting rid of dead birds and the demand on the composting system.

The ripple effect of a cholera break of this magnitude included the following:

- Additional work and stress of having to "manage" cholera-positive flocks and prevent spread to other farms;
- Antibiotics at least every two weeks and the search for one that would actually be effective;
- Dead bird disposal. Debate over rendering them, burying them, or how to dispose of quickly and safely;
- How to eliminate an endemically increasing population of rodents and wildlife;
- Cleaning up cholera positive barns and the additional work to remove semi composted cholera positive material in order to clean up the entire farm;

- Need to locate and molt clean flocks, which requires barn space, time, bird transport, and considerable oversight to ensure it is done without compromising bird welfare; and
- High mortality resulted in less birds in the barns which equated to less body heat produced. The lack of supplemental heat overlapped with colder weather which resulted in poorer environmental conditions and uncomfortable flocks.

Cholera is one disease agent that flared up in several companies. Others included mycoplasmas, both synoviae and gallisepticum, and coronavirus in locations where these agents had not been reported in many years. The common element they all share is ineffective biosecurity. We must never lose sight of the fact that biosecurity only works when we have the people in place and with the mandate and the resources to make it happen. These disease breaks were a direct reflection of lack of human resources both to implement the program and to monitor them to ensure compliance. The bottom line is that people and companies did the best they could with all the constraints which were present.

Routine technical support provided by allied industry technical representatives, veterinarians, and consultants came to a halt due to travel restrictions. In addition, especially during other crises, these individuals were helpful in supporting the activities of the company veterinarian and managers. With COVID-19, virtual assistance to troubleshoot, train, and audit became necessary. Although virtual help has its limitations, it did cause allied industry to become more creative and flexible and allowed companies to get specific assistance. Many retailers were accepting of virtual animal welfare audits for example. Doing real-time assessments was at the mercy of internet capabilities, cell phone reception, and sometimes amount of light within barns. A more reliable solution was to capture photos and videos to verify processes in place in hatcheries on farms to replace in-person visits but these took an extraordinary amount of time, and the realization that it was a learning process. Some auditable criteria needed to be adapted from in-person to virtual.

Veterinarians became more involved in working with health and safety managers to prevent spread within a facility by helping to design programs and educating employees. Despite best efforts however, in some cases employees were not as concerned about social distancing as what managers were. In one hatchery, managers carefully marked on the lunch room floor where all chairs should be to maintain six feet of physical separation. Within five minutes of break time, the employees pulled all the chairs back together around one table.

Lastly, I need to mention the impact of this virus on turkey prices and turkey demand. Even before the COVID-19 pandemic struck, the turkey industry was struggling. The industry was faced with an oversupply situation, and many of the country's top turkey companies made adjustments to find balance. U.S. turkey production decreased 2.7% in 2019 from its 2018 levels, which incidentally were about 1.3% lower than the 2017 levels. Dr. Thomas Elam, president of Farm Econ LLC, reported that the average retail price of turkey, whole turkeys, frozen whole turkeys, in grocery stores in 2020 was down 7% from 2019, which is the lowest retail price in a decade (1). After Thanksgiving, I bought two 21 lb. frozen turkeys for less than $10. This was the grocery chain's solution to clear the remaining large size turkeys left, as most consumers bought the smallest turkeys, they could find due to fewer people around the dinner table during the holidays.

The National Turkey Federation commented in fall 2020, that it was clear that disruptions to foodservice have been significant and will continue to have an impact on the turkey industry as we look toward 2021. Some projections put overall turkey foodservice losses over 20 percent, and it will take time to rebuild that business as the economy reopens. While we've seen consistent gains at retail, with ground turkey performing especially well, it is important to remember that growth at the supermarket cannot fully offset the losses at foodservice. It is too early to tell how all of this will affect turkey production, but it is reasonable to assume some companies have had to make cutbacks to reflect the lost foodservice business and that additional reductions may be on the horizon in 2021, at least until the virus is fully contained (6).

A survey of companies in the Minnesota food and agriculture manufacturers and processors indicated that while COVID disrupted food and agriculture supply chains, they did not break. Companies used creative, innovative strategies to stabilize their businesses. The disruptions exposed critical gaps that provide insight into how to support supply chain resiliency with pain points listed in Table 1 below (7).

There is no doubt that COVID-19 impacted all of us in ways that tested our resilience, our adaptability, and our ability to find and implement solutions that best fit each new challenging circumstance that we faced on a daily basis. Unfortunately, this paper could not be a Lessons Learned regarding COVD 19 as we are still in the midst of navigating our ways through this pandemic both personally and professionally.

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Table 1. Pain points.

Pain Points		
Barriers	**Supply Chain Disruptions**	
· National and State effects · Varying state criteria · Shutdowns · Short planning window for reopening · Fearful public · Unemployment higher than wages	**Labor Shortages** · COVID exposure · Absenteeism	**Safety Lags** · Lack of affordable PPE and cleaning supplies. · Lags in COVID testing
	Market/Sales Losses · Cancelled events · Overnight demand shift in foodservice needs	**Inconsistent Inputs** · Limited, overpriced PPE · Limited inputs and packaging
	Bottlenecks · Meat plant shutdowns and backlogs · High demand in grocery outlets	**Difficult Business Decisions** · Shifts: e-commerce, new products/markets, plant retrofits · Shutdowns, furloughs & closures

OCCURENCE OF A NOVEL VIRULENT MAREK'S DISEASE VIRUS IN BROILER BREEDER CHICKEN FLOCKS IN POLAND

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SUMMARY

Marek's disease (MD) presents an economically important threat to international poultry production. In spite of protective vaccination programs, the evolving pathogenicity of field Marek's disease virus (MDV) causes a serious loss in the production of broiler, layer and breeder chickens. Currently in Poland the vaccination practices are focused on vaccination *in ovo* of one-day-old broiler chickens with the vaccine based on the herpesvirus of turkey (HVT) (*Melagrid herpesvirus-1*) and alternatively CVI/988 Rispens serotype-1 attenuated vaccine. The layer and breeder chickens are preferably vaccinated with CVI988/Rispens vaccine or bivalent Rispens plus HVT-based vaccines. Recent reports between 2015 and 2017 from chicken flocks in Poland indicated the higher incidence of MD in broiler flocks, probably because of the HVT vaccine failures.

In 2020 and 2021, the occurrence of MD has been noted among at least three flocks of broiler breeder chickens aging from 20 to 23 weeks in western Poland, showing increased mortality, reaching 20 percent of the flock. The vaccination manner included *in ovo* administration of Rispens vaccine and then Rispens and vectored HVT+ Newcastle disease vaccination of one-day-old chicks. The drop in egg production was also registered. During postmortem, the birds showed enlargement of the liver, spleen, and proventriculus with greyish and flat tumor presence. Laboratory analysis focused on reverse transcription polymerase chain reaction (RT-PCR) focused on avian leukosis types A, J and K showed negative results. The negative results were obtained by RT-PCR for avian reticuloendotheliosis virus (REV) also. Inversely, the real-time PCR conducted in Poultry Science Unit at the University of Georgia (Athens, GA, United States of America) showed the DNA presence of Rispens and HVT vaccine strains parallelly with DNA of the virulent MDV-1 strain. Furthermore, metagenomic analysis conducted by next-generation sequencing technology (NGS, Ilumina) showed the highest nucleotide similarity (96.0 percent identity) of a novel MDV isolate to previously sequenced strain Polen5 investigated during 2010 in Germany.

Taking into account the inconclusive MD course as well as the potential failure in vaccination using highly effective Rispens and bivalent vaccines, this particular issue should be further investigated and resolved.

INTRODUCTION

MD presents one of the major concerns in poultry production worldwide (3, 6, 11, 13, 15, 18, 23). As a contagious and fatal disease of poultry it presents one of the most serious threats for commercial production of chickens (5, 22). The economical constrains due to the high mortality of chickens is spite of the applied vaccination cost US $1-2 billion annually (15). The other losses are caused by egg drop production. Previous reports indicate the possibility of MD occurrence in turkey and Japanese quail flocks. In spite of the fact that MD was firstly described in 1907 by Dr József Marek, who identified the clinical signs, including polyneuritis signs with histopathological lesions in brain and peripherial nerves of chickens, the disease still presents a problem for massive poultry production (1, 2, 4). The current name of 'Marek's disease' has been introduced in the 1960s by Dr Biggs, who observed clinical signs and lesions similar to those during lymphoid leukosis (LL) (4-5,2). MDV, the so-called Gallid *herpesvirus 2* (GaHV-2), represents an etiological agent of MD showing a strict dependence on viable cells and the requirement of MDV strains and vaccine stock storage in liquid nitrogen tanks (-196ºC) and the continuous cold-chain administration of the vaccine. Three serotypes of MDV are distinguished, including virulent and oncogenic strains belonging to serotype 2 (MDV-1), non-pathogenic serotype 2 (MDV-2) and serotype 3 strains (MDV-3), also called HVT or *Melagrid herpesvirus-1* (MeHV-1). Meanwhile, the protective vaccination includes vaccination of broiler chickens with FC126 HVT vaccine and layer and breeder chickens with attenuated CVI988/Rispens strain, and/or bivalent vaccines based on Rispens and HVT or

HVT and SB-1 strain, belonging to the MDV-2 serotype (3, 16, 19, 20).

During MD course, different form of the disease could be observed, including acute and classical forms. The application of protective vaccination in Europe during the 70s of the 20th century has limited the occurrence of acute MDV form (3, 16, 25). However, as a consequence of MDV evolution, four pathotypes of the virus could be distinguished, including mildly virulent MDV (mMDV), virulent MDV (vMDV), very virulent (vvMDV) and very virulent plus strains (vv+MDV), which are known to break the protection elicited by vaccination (10, 21, 24). An additional complication in MD diagnosis are caused by the co-infection with MDV-1 and avian leukosis (ALV) or reticuloendotheliosis (REV) virus (9). Secondly, interactions with other co-infecting viral agents, including chicken anemia virus (CIAV), fowl adenoviruses (FadV) or chicken or infectious bursa disease virus (IBDV), are known (12, 17). In terms of MD vaccination failures, it seems that vvMDV or vv+MDV are derived from less virulent strains as an effect of the massive vaccination of chickens.

The aim of our study was to investigate the epidemiology of the increased mortality and the observed tumoral disease observed among broiler breeder flocks in Poland between 2020 and 2021.

MATERIALS AND METHODS

Chicken flocks. The broiler breeder chickens aged from 20-23 weeks-old originated from three different farms suspected of MD, LL or ALV. The farms were located in western Poland and their size ranged from 10.000 up to 50.000 birds. The vaccination manner of broiler breeders included the *in ovo* administration of Rispens vaccine, then Rispens and vectored HVT+ Newcastle disease vaccination of one-day-old chicks. During postmortem, the birds were examined for the presence of gross lesions in the internal organs. Sections of liver and spleen were collected for further virus isolation and molecular biology examinations.

Strains. Attempts to isolate MDV in chicken embryo fibroblasts (CEFs), prepared from SPF chicken embryos, have been made (LTZ, Cuxhaven, Germany). MDV strains were incubated at 37.8°C in 5% CO_2 until cytopathic effect (CPE) was observed.

DNA and RNA extraction. The DNA and RNA from sections of the internal organs of chickens, as well as cell culture stocks, were extracted from 200µL of the virus, using a QIAamp Mini Kit and a RNasy Mini Kit (Qiagen, Hilden, Germany), according to the producer recommendations. Negative controls for real-time PCR were DNA or RNA extracted from noninfected SPF chicken embryo fibroblasts.

DNA controls. The DNA of the attenuated CVI988/Ripens and FC126 HVT strains were extracted from commercial vaccine (MSD Animal Health, Boxmeer, the Netherlands). Reference strain: RB1B was also used as a positive control for MDV-1, the panel of positive reference RNA for ALV types A, J and K has been also applied (Merck-Intervet, Poland).

Real-time RT-PCR for ALV type A, J, K and REV. The assays were conducted in a ABI 7500 system (Applied Biosystems, Foster City, California, United States of America). The reactions were performed using primers and probes previously described (7, 8, 14). All conditions of real-time PCR assays were consistent with the ones referred to in literature (7, 8, 14).

Real-time for MDV-1, MDV-2, HVT and CVI988/Rispens. The assays were conducted in ABI 7500. All assays were consistent with protocols described by Woźniakowski *et al.* (25) and Niczyporuk J. (17). All conditions were following the recommendations from the referred papers. The PCR examinations have been performed by the Poultry Science Unit at the University of Georgia (Athens, GA, United States of America).

Next generation sequencing. Three CEF cell-cultured isolates of MDV-1, originating from broiler breeder farms, were subjected to NGS sequencing. The third passage of CEFs infected with isolates was used to purify the viral DNA. The cell supernatants were subjected to filtration (0.22 µm syringe filter) and precipitated by addition of 20% Polyethylene glycol (PEG) and 2.5M sodium chloride. The solution was agitated for 12 h at 4°C. The solutions containing virions were then pelleted by centrifugation at 18000 g for 60 min at 4°C, resuspended in Minimal Essential Medium (MEM) and treated with DNAse I (ThermoFisher Scientific). The DNA of MDV was further extracted using QIAamp Mini Kit and a RNasy Mini Kit (Qiagen, Hilden, Germany)

DNA libraries preparation. The DNA libraries were prepared with NEBNext® UltraII DNA Library Prep Kit for Illumina (New England Biolabs, Ipswitch, United States of America), and sequenced on MiSeq instrument (Illumina) in PE250 mode. In total 100,516 reads have been obtained. The mean coverage of the whole MDV sequences ranged from 10 to 40 times. The complete sequence sequence has been aligned using BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Clinical observations. The observed mortality among the affected flocks of broiler breeders reached maximally 20 percent. No specific clinical signs have been observed except a drop in egg production. The affected chicken flocks occurred in 2020 and 2021 in Western Poland. The chicken age ranged from 20 to 23 weeks-old. During the conducted post-mortem of dead animals the MD specific enlargement of the liver and spleen has been observed with the presence of flat tumors in the liver, as well as the marble structure in the spleen (Figures 1A, B). The proventiculus was swollen and the tumoral changes have been observed in the ovaries. The laying of eggs was abrogated since a number of breeder hens died before laying. Interestingly, the tumoral changes have been also observed in breast muscles of chickens (Fig.1C).

Real-time RT-PCR for ALV type A, J, K and REV. The results of all conducted RT-PCRs specific for ALV-A, J, K, as well as REV presence, in the sections of the diseased chickens' internal organs were negative.

R**eal-time for MDV-1, MDV-2, HVT and CVI988/Rispens.** The results of PCRs specific for MDV revealed the presence of virulent MDV-1 (cycle threshold value C_T = 18.0, as well as MDV-2 and (C_T = 23.2) and HVT (C_T = 34.2), but the results for CVI988/Rispens were negative (C_T = 40.0).

Next generation sequencing (NGS) The conducted next generation sequencing of the infectious material originating from broiler breeders showed the presence of the genomic sequence of MDV-1 with the similarity of 96.0 percent to previously sequenced Polen5 strain processed by scientists in Germany (Figure 2).

DISCUSSION

MDV infection may cause different clinical signs and lesions which may indicate lymphoid leukosis (LL) or reticuloendotheliosis (RE) (1, 4,8, 9, 25). In terms of clinical signs, the most common ones include leg paralysis, torticollis, and depression. Most frequently paralysis or torticollis occurs after 2-12 weeks post-infection (1, 5, 10, 22). Taking into account the previous international reports, the MD occurred in chickens over three weeks of age (22, 25). The late MD outbreaks are observed in 8-9 week-old chickens. The mortality may reach up to 60 percent, but in obvious observation it does not exceed 20 percent (22, 24). The mortality is directly related to the protective vaccination delivered to chickens, which in majority protects against the clinical MD onset but not from subsequent infection with virulent MDV. Similarly, in the case of MD emergence in broiler breeder chicken flocks the disease has occurred in double-vaccinated chickens aged from 20 to 23 weeks.

In terms of the observed lesions the visceral lymphomas may occur in different organs including the spleen (marble spleen), liver (with flat or nodular gray tumors), ovaries, testis, proventriculus, heart, intestines, kidneys, and skeletal muscles (10, 22, 24). Similarly, to the classical description of visceral lymphomas among the affected flocks of chickens in Poland the presence of tumoral lesions in the liver and spleen have been observed. Interestingly, in previous reports from Poland and other European countries the lesions in skeletal muscles were seldom (25). During the presented study the presence of tumors were observed in breast muscles of the infected chickens. This may be referred to the emergence of MD in chickens older than 20 weeks, what facilitates infiltration of feather follicles and finally skeletal muscles. During the past 25 years the nodular erythematous tumors was detected especially in feather tracts of chickens and were termed as 'Alabama Red Leg syndrome' (personal communication).

The conducted differential diagnosis for other potential agents (ALV A, J and K or REV) revealed that the exclusive etiological agent of the observed disease in broiler breeder chickens was MD. The further PCR examination specific for MDV-1, MDV-2, HVT and CVI988/Rispens may suggest that the conducted double-vaccination of chicks by *in ovo* route, then at the 1st day of life may lead to overloading of immune system and results in abrogation of CVI988/Rispens replication. This may also lead to a lack of long-term protective immunity elicited by the attenuated MDV-1 strains against vvMDV.

The conducted full-genome sequencing of the isolated MDV-1 suggests its high genetic homology to the recently isolated Polen5 strain, which may circulate in chicken populations in Poland and the rest of Eastern Europe.

CONCLUSIONS

Concluding the conducted study, we have revealed the recently occurring MDV-1 virulent strain in broiler breeder flocks. The direct reason for the lack of protective immunity could be the overloading of the chick immune system by two-step vaccination by *in ovo* route and just after hatching. Other studies are required to fully elucidate the cause of CVI988/Rispens replication failures which probably lead to non-efficient protection of breeder or layer hens.

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Figure 1. Post-mortem conducted on broiler breeder chickens in age from 20-23 weeks old originated from three different farms suspected to be MD or LL affected. (A) tumoral lesions in liver, (B) enlarged spleen with tumoral marble structure, (C) tumor in breast muscles.

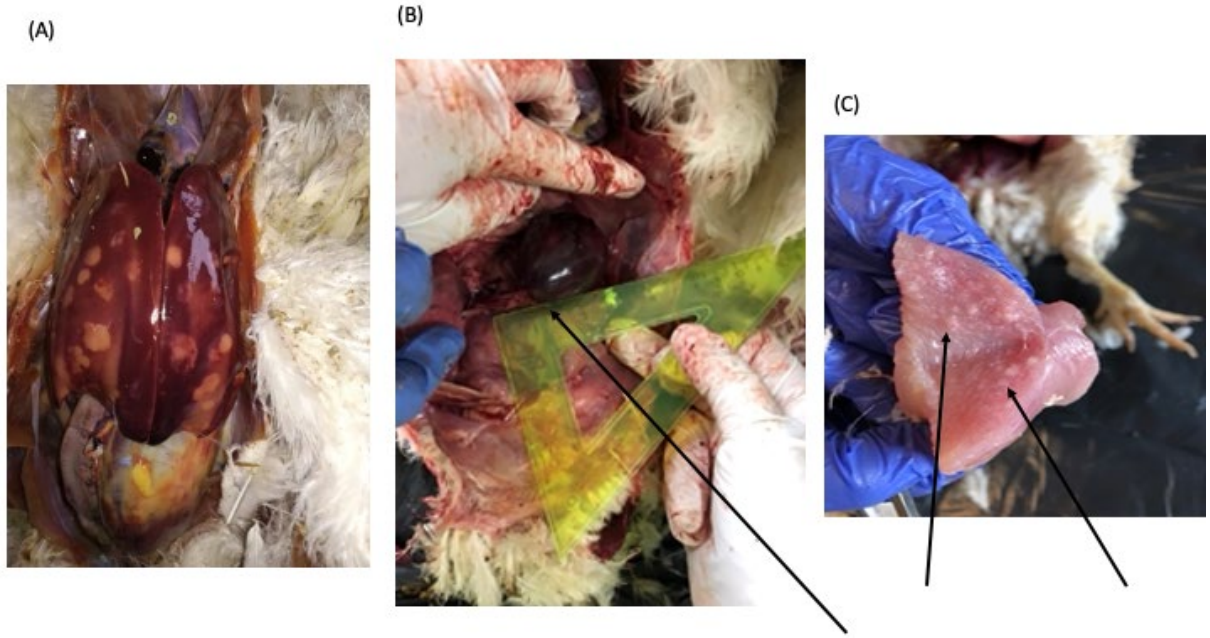
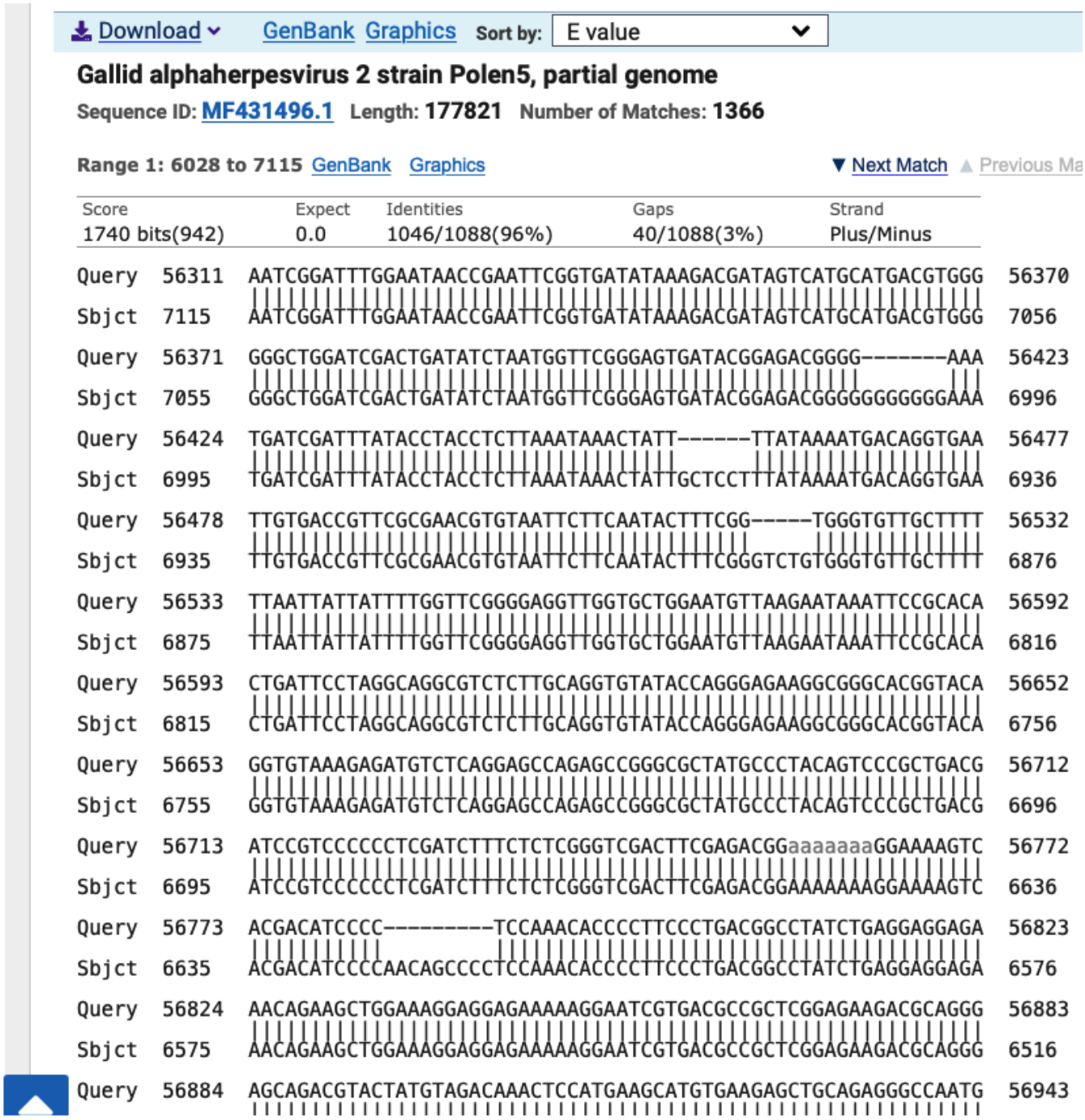


Figure 2. The results of next generation sequencing of MDV-1 isolates originating from MD outbreaks in broiler breeder flocks in Poland. The sequence has been aligned using BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).



EFFECTIVENESS OF THE ANTHELMINTIC LEVAMISOLE IN REDUCING ROUNDWORM (*ASCARIDIA GALLI*) INFECTION IN BROILER CHICKENS

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SUMMARY

The nematode parasite *Ascaridia galli* is a common parasite of chickens; however, approved treatment options are limited. Many of the available chemotherapeutics require an extra-label use under a veterinary prescription. This study was conducted to evaluate the efficacy of the anthelmintic levamisole in reducing the number of *A. galli* in broiler chickens. Broiler chickens were challenged with a field strain of *A. galli* isolated from broilers in north Georgia. The challenge consisted of 100 infective ova per bird for six days, starting on Day 6 of the study. Treatment regimens were either a single administration of levamisole at 25 mg/kg on day 29, or daily treatments at 5 mg/kg on days 29 – 33. The one-day regimen significantly reduced *A. galli* numbers to extremely low levels. The five-day treatment of 5 mg/kg/day significantly lowered *A. galli* numbers from the challenge control, but not as low as the one-day treatment. Zootechnical performance was evaluated, however, no significant differences were present.

INTRODUCTION

Ascaridia galli is one of the most common parasitic roundworms of poultry, occurring in both chickens and turkeys (1). Although indoor rearing of commercial poultry and the shortened growing period for broiler chickens reduces the opportunity for exposure to worms, regular programs of de-worming are recommended for poultry that are to be kept for more than a few weeks (2). Pathogenicity of ascarids is typically low, although heavy infections can cause anemia, diarrhea, anorexia, and weight loss (3). Currently, there are only two compounds approved to treat roundworms in chickens, and one has been found to be largely ineffective (2). Other anthelminthic products are commercially available, but are not labeled for poultry, so must be used off-label with a veterinary prescription (4).

This study was designed to determine if the anthelminthic levamisole, administered for one day at 25 mg/kg, or at a lower dose of 5 mg/kg/day over five days, would reduce the worm burden of *Ascaridia galli* in broiler chickens, as well as to assess whether treatment with levamisole would affect broiler performance.

MATERIALS AND METHODS

Birds and housing. Male commercial meat-type chicks were placed at day-of-hatch into 5 x 10 feet floor pens (stocking density of 1.0 feet^2 per bird) with approximately four inches of fresh pine shavings (at placement), in a solid-sided barn, with concrete floors under ambient humidity. Birds were sprayed with a commercial coccidiosis vaccine (Advent^{TM}, Huvepharma, Peachtree City, GA) at the recommended dose at placement. Litter was not replaced or amended during this study. Feed and water were available ad libitum throughout the trial.

Bird allocation and pen randomization. Three thousand male chicks were assigned to six treatment groups (500 birds in each group) with 10 replicate pens per treatment and 50 birds per pen. The pen facility was divided into 10 blocks with each block containing each of the six treatment groups. The treatment groups (TG) were as follows (Table 1): TG1-nonmedicated/nonchallenged; TG2-nonmedicated/challenged; TG3 and TG5-one day medicated; TG4 and TG6-five days medicated. Treatment groups were assigned to pens using randomized complete block. Random permutation tables (5) were used to complete randomization and assign treatment groups to pens. The study began when birds were placed (day-of-hatch; Day 0), at which time birds were allocated to experimental pens. Only healthy birds were selected. No birds were replaced during the course of the study.

Ascarid egg challenge. The birds were challenged with a field strain of *A. galli* isolated from a north Georgia broiler farm. The challenge was 100 infective ova per bird, given in the drinking water, to TG2, TG5 and TG6 for six days starting on day six of the study.

Levamisole treatment. On Day 28, the average body weight of the birds was determined and the dose

per treatment was calculated. The treatment was administered in the drinking water, with TG3 and TG5 receiving 25 mg/kg of levamisole (Prohibit^{TM}, Huvepharma, Peachtree City, GA) on Day 29 only, and TG4 and TG6 receiving 5 mg/kg of levamisole each day from Day 29 through Day 33. All of the medicated water was consumed during the treatment period and fresh water was provided after consumption of the medicated water each day.

Ascarid enumeration. On Day 28, one bird per pen was weighed and the entire intestinal tract (below the gizzard to the vent) was removed and examined. The number of ascarids present in each intestinal tract was recorded, and the mean number of ascarids from all 10 pens in each treatment group was calculated. On Day 40, 15 birds per pen were weighed and the entire intestinal tract was removed. The number of ascarids present in each intestinal tract was recorded, and the mean for the 15 birds per pen was calculated. Then, the mean number of ascarids from all 10 pens in each treatment group was calculated.

Body and feed weight. All birds were weighed by pen on Days 0, 28, 35, and 40. Feed added to each pen's feeder was weighed at the beginning of each formulation period on Day 0, 28, and 35 (starter and grower respectively). Any additional bags of feed were weighed for each pen during each formulation period. Feed was distributed as needed to feeders from pre-weighed bags throughout each period. Feed remaining in feeders (and feed bags if applicable) was weighed and disposed of on Day 28, 35 and 40. Empty pen feeder weights were recorded prior to study initiation. Date and removal weight (kg) were recorded on all birds culled (or found dead). The trial was terminated on Day 40.

Data analysis. Means for ascarid counts, weight gain, feed consumption, feed conversion ((adjusted for mortality): (feed consumed/ final live weight + mortality weight)), and cause of mortality were calculated. The mortality was assessed by gross lesions on necropsy. Statistical evaluation of the data was performed using STATISTIX for Windows program (Analytical Software, Tallassee, FL). The procedures used were general linear procedures using ANOVA with a comparison of means using least significant difference [(t-test) (LSD) (T)] at a significance level of 0.05.

RESULTS

There were no significant differences in ascarid numbers on Day 40 and Day 28 (pre-treatment) in the nonmedicated, challenged control group, T2 (Table 2). Since all pens were randomized and despite boot changes between all pens, there were some pens that were not ascarid challenged pens that had a small number of ascarids present. On Day 40, the challenged control (T2) had a significantly higher ascarid number per bird (72.33^{A}) than any other treatment (Table 2). There was a significant reduction in ascarid number for the one-day treatment at 25 mg/kg (T5), compared to the challenge control, T2 (one-day 0.07^{C} versus control 72.33^{A}). There were no significant differences noted between the one-day treatment (T5) and the three treatments that were not challenged with ascarids (T1, T3, T4). The five-day treatment at 5mg/kg (T6) also had a significantly lower ascarid count than the control, T2 (five-day 25.97^{B}versus 72.33^{A} control). The five-day treatment (T6) had higher ascarid counts than the one-day treatment, T5 (25.97^{B} five-day versus one-day 0.07^{C}).

There were no significant differences in any performance parameter at either 28 or 35 days (data not shown). On day 40, the one-day treatment had a non-adjusted FCR that was significantly higher than the non-challenged; however, there was not a difference in FCR to any of the challenged treatments, nor was there any significant difference once the FCR was adjusted for mortality.

DISCUSSION

Ascarid infection in poultry is a common problem that needs more solutions. Currently, there are very few anthelminthic options available for poultry practitioners, and overuse may cause worms to build resistance against these drugs (4). The results presented here indicate that levamisole is effective in reducing *A. galli* infection in broiler chickens. Moreover, treatment with levamisole did not have any negative impact on bird performance. The one-day treatment of 25 mg/kg significantly reduced the worm burden to extremely low numbers, while the five-day treatment of 5 mg/kg/day significantly lowered ascarid numbers from the challenge control, but not as low as the one-day treatment. Future studies are needed to evaluate the efficacy of levamisole against other species of worm parasites in chickens.

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Table 1. Experimental design of the study.

ID	Treatment Description	Ascarid Challenge?	Pens/Treatment	Birds/Pen
T1	Nonmedicated (Negative Control)	NO	10	50
T2	Nonmedicated (Challenge Control)	YES	10	50
T3	One (1) Day Medicated*	NO	10	50
T4	Five (5) Day Medicated**	NO	10	50
T5	One (1) Day Medicated*	YES	10	50
T6	Five (5) Day Medicated**	YES	10	50
*Dose at Day 29 for one (1) day medication was 25 mg/kg continuously in water. ** Dose at Day 29 to Day 33 for five (5) day medication was 5 mg/kg per day continuously in water.				

Table 2. Results.

Treatment	Day 28 ascarid count (single bird per pen)	Day 40 ascarid count (mean of 15 birds per pen)
1. Nonmedicated (negative control); no ascarid challenge	0.90^{B}	0.13^{C}
2. Nonmedicated (challenge control); ascarid challenge	57.00^{A}	72.33^{A}
3. One-day medicated; no ascarid challenge	0.30^{B}	0.03^{C}
4. Five-day medicated; no ascarid challenge	0.30^{B}	0.02^{C}
5. One-day medicated; ascarid challenge	72.20^{A}	0.07^{C}
6. Five-day medicated ascarid challenge	63.60^{A}	25.97^{B}