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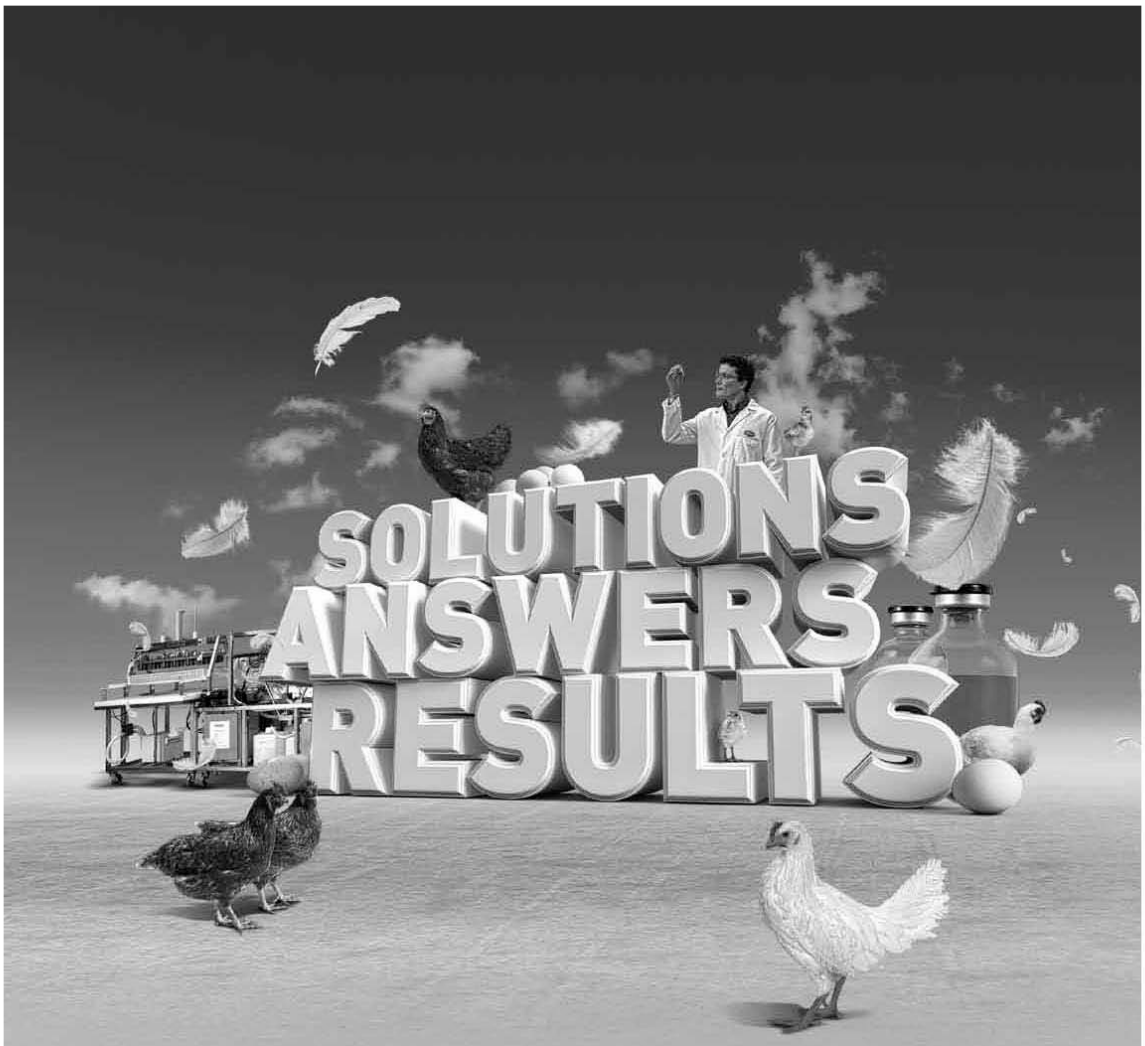
PROCEEDINGS OF THE SIXTY-FIRST WESTERN POULTRY DISEASE CONFERENCE

April 2-4, 2012 Scottsdale, AZ



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SPECIAL ACKNOWLEDGMENTS

The 61st Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. More than 40 organizations, companies and individuals have once again given substantial financial support. Many companies and organizations, including some that also contribute financially, send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

The WPDC is extremely honored to give a special acknowledgement to **Pfizer Animal Health** who contributed at the Super Sponsors level by sponsoring our electronic proceedings. In addition, WPDC is pleased to acknowledge our Benefactor contributors, **American Association of Avian Pathologists, Inc.**, and **Merck Animal Health.** Once again, the WPDC is forever grateful to our distinguished Patrons, Donors, Sustaining Members, and Friends of the Conference who are just as important in making the conference a success. All our contributors and supporters are listed on the following pages. We greatly appreciate their generosity and sincerely thank them and their representatives for supporting the WPDC.

Many have provided special services that contribute to the continued success of this conference. For this year's meeting, the WPDC has contracted Conference and Events Services, of the University of California, Davis, for providing budgetary and registration support for the conference. We would like to thank Ms. Teresa Brown for her exceptional work with our conference.

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. We again acknowledge and thank *Omnipress* (Madison, WI) for the handling and printing of this year's Proceedings. Once again, we acknowledge Bruce Patrick (Graphic Communications, Brigham Young University) for the cover design of the printed proceedings.

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61st WPDC PROCEEDINGS

The Proceedings of the 61st Western Poultry Disease Conference are <u>not</u> 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. Copies of the Proceedings are available in either book or electronic formats.

Copies of these Proceedings are available from:
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18th WPDC – 1969	R. McCapes	Dean C. Young		
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58th WPDC – 2009	R. Crespo	Victoria Bowes		G.L. Cooper
59th WPDC - 2010	V. Bowes	Nancy Reimers		
60th WPDC - 2011	N. Reimers	Larry Allen		John Robinson
61st WPDC - 2012	L. Allen	Vern Christensen		
62nd WPDC - 2013	V. Christensen	Portia Cortes		

MINUTES OF THE 60TH WPDC ANNUAL BUSINESS MEETING

President Reimers called the meeting to order on Monday, March 21, 2011, at 5:10 PM, at the Holiday Inn Capitol Plaza, Sacramento, CA. There were 26 people in attendance.

APPROVAL OF 59th WPDC BUSINESS MEETING MINUTES

The minutes from the 59th WPDC business meeting were reviewed and a motion was carried to approve them as printed in the Proceedings of the 60th WPDC.

ANNOUNCEMENTS

Dr. Reimers acknowledged all the contributors; in particular, Pfizer Poultry Health Division which contributed at the Super Sponsor level by sponsoring the flash drives of the proceedings. In addition, Dr. Reimers acknowledged those at the Benefactor level, which included the American Association of Avian Pathologists, Intervet/Schering-Plough Animal Health and Merial Select. She also thanked all the contributors for their generous donations. The efforts of the current WPDC officers were acknowledged for their work and participation in the organization of this year's meeting.

REPORT OF THE SECRETARY-TREASURER

Dr. R. Chin presented the Secretary-Treasurer report. As predicted, there was an overall loss of $10,473 for the 59th WPDC. Most of this was due to hotel costs at the Fairmont, which almost doubled our normal cost at the Holiday Inn in Sacramento, and the poor money exchange rate. In addition, there were additional costs for travel. On the positive side, our contributions again for Canada were excellent with $34,650 in contributions. Currently, WPDC has $51,127 in savings.

Contributions for this year's meeting (60th WPDC) are outstanding at $38,370 which is by far the most we have had in contributions for numerous years. With this outstanding amount of contribution, lower cost for the meeting in Sacramento, and more thriftiness by the Executive Committee, it appears that WPDC will make up what was lost last year.

It was recommended that registration fees stay the same for next year's meeting in Arizona, i.e., $175 for presenters and $250 for non-presenter, advance registration. (Note: this does not include the ACPV workshop.)

In addition, the levels of contributions and their entitlements will remain the same. Hence, for Friends of the Conference ($100-$249), no reduced or complimentary registrations; for Sustaining Members ($250-$499), one reduced registration; for Donors ($500-$999), one complimentary registration; and for Patrons ($1000-$2999), two complimentary registrations. Benefactors ($3000-$4999) and Super Sponsors ($\geq$$5000) will receive their current entitlements of 4 and 5 complimentary registrations, respectively, and additional proceedings.

There were no objections to paying for WPDC officers' expenses if they needed it to attend.

REPORT OF THE PROCEEDINGS EDITOR

Dr. David Frame presented the Proceedings Editor report. There are a total of 68 papers in the proceedings. One presenter did not submit a paper. (Ed. The presenter from Libya was unable to obtain a visa and cancelled at the last minute, and the presenter from Iran was a no-show.) The flash drives were again sponsored by Pfizer Poultry Health Division.

NEW BUSINESS

In 2012, the 61st WPDC will be in Scottsdale, AZ, at the DoubleTree Resort by Hilton Hotel Paradise Valley, April 2-4, 2012. ACPV will sponsor a workshop on Sunday, April 1, 2012.

Dr. Reimers reported that the WPDC Executive Committee nominated Dr. Portia Cortes for Program Chair for the 62nd WPDC in 2013. There were no other nominations and Dr. Cortes was elected unanimously as program chair-elect.

Dr. Reimers nominated the following officers for 2011-2012:

Program Chair: Dr. Vern Christensen
President: Dr. Larry Allen
Past-President: Dr. Nancy Reimers
Contributions Chair: Dr. Yan Ghazikhanian
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Richard Chin
Program Chair-elect: Dr. Portia Cortes

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

It was approved that the 62nd WPDC will be in Sacramento, CA, at the Holiday Inn Capitol Plaza. Tentative dates are March 24-27, 2013. (Ed. These dates have been confirmed.)

Dr. Reimers said that ANECA has extended an invitation to hold the 63rd WPDC with ANECA in Mexico, tentatively April 2-5, 2014. ANECA representatives met with the WPDC Executive Committee on Sunday and discussed this invitation. WPDC Executive Committee agreed to hold the next joint meeting in 2014 in Puerto Vallarta, with Dr. Ernesto Soto as the sole program chair. He will ask ANECA and WPDC members to form a committee to assist him in the program development. There were no objections.

The 64th WPDC, in 2015, will tentatively be back in Sacramento, CA, at the Holiday Inn Capitol Plaza.

Dr. Reimers mentioned that CE credits will be emailed to everyone by the AAAP office. There were no additional items for discussion.

Dr. Reimers turned the presidency over to Dr. Larry Allen who adjourned the meeting at 5:35 PM.

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PROCEEDINGS OF THE SIXTY-FIRST WESTERN POULTRY DISEASE CONFERENCE

DISEASES OF PIGEONS AS SEEN AT CAHFS IN THE LAST DECADE

J. Kelly, B. Charlton, and C. Sentíes-Cué

CAHFS Turlock, PO Box 1522, Turlock, CA 95381

SUMMARY

Pigeons have played a significant role in several of history's greatest societies and wars, including World War II. They provided food, fertilizer, and, perhaps, most importantly, messages. Today, the same qualities that were valued in the past, such as speed and conformation, are still appreciated. Currently, some of the most common uses for pigeons include racing, showing, and food. Yet, for as much as we have relied upon pigeons, we still know relatively little about pigeon diseases and disease trends.

In this retrospective study, a query was performed on the records of all the California Animal Health and Food Safety (CAHFS) Laboratories for all pigeon necropsy cases from August of 2001 to August of 2011. Some cases, submitted for exotic Newcastle disease surveillance in 2002 and 2003, were eliminated from the study. Once this data set was obtained, the cases were categorized based on the listed diagnosis.

While an etiologic diagnosis was preferred, some cases had only a morphological diagnosis and others had no diagnosis at all. Once these cases were categorized, the most common diseases underwent further investigation to determine trends in season, location, or bird purpose.

RESULTS

A total of 571 cases were identified for this study. They were first categorized based on their etiology. Viral etiologies were the most commonly diagnosed at 45.01%. That was followed by parasitic, 36.43%, bacterial, 27.67%, and fungal, 12.61%, etiologies. They were then broken down further by specific agents. The most common agents and their prevalence are listed below in Table 1. Other identified diagnosis, such as trauma, toxicities, and nutritional imbalances, were also identified and accounted for 12.96% of the total cases. Other cases only had a morphological diagnosis and accounted for 13.66%.

Table 1. The percentage of the most common diseases of pigeons seen at CAHFS broken down by etiological diagnosis.

Viral	%	Parasitic	%	Bacterial	%	Fungal	%
Paramyxovirus-1	22.07	Trichomoniasis	16.11	*S.* Typhimurium	6.13	Aspergillosis	7.18
Circovirus	15.06	Coccidiosis	3.68	*E. coli*	6.13	Candidiasis	1.58
Other viral	7.88	Other parasitic	16.64	Other bacterial	15.41	Other fungal	3.85
Total viral	45.01	Total parasitic	36.43	Total bacterial	27.67	Total fungal	12.61

OUTBREAK OF EASTERN EQUINE ENCEPHALITIS IN RING-NECK PHEASANTS IN ONTARIO

M. Brash, M. MacAlpine, J. DeLay, and D. Ojkic
Animal Health Laboratory, University of Guelph, Ontario, N1H 6R8, Canada

CASE REPORT

In early October 2011, an upland game hunting reserve experienced increased morbidity in one of six fly pens of ring-necked pheasants (*Phasianus colchicus*). This particular pen, located 50 meters from a hardwood swamp, had been stocked with approximately 3,200 nine week birds, 11 weeks previously. Birds were noted to be dull and depressed, weak and uncoordinated, and many birds had loss or breakage of feathers on their backs and tails with hemorrhage from the broken tail feathers. Despite the initiation of intensive culling, clinical signs did not resolve and three days later, dead, live sick and live healthy pheasants were submitted to the Animal Health Laboratory, University of Guelph for necropsy.

Necropsy findings in the dead pheasants varied and included mild focal epicardial and pulmonary hemorrhage in bird number one, excessive blood-tinged fluid in the small intestine with serosal congestion in bird number two, and cardiac enlargement, right ventricular dilation, increased amounts of pericardial fluid and an enlarged, mottled liver in bird number three. Other than multifocal hemorrhage of muscles of the back of the head and the legs, there were no significant lesions in the live sick pheasants. The live healthy pheasants had no gross lesions.

Histologically, two of the three dead pheasants and all three of the live sick pheasants had meningoencephalitis with low to moderate numbers of plasma cells, lymphocytes and heterophils within the meninges with multifocal gliosis, mild loss of Purkinje cells, neuronal necrosis, and narrow predominantly lymphoid perivascular cuffs encircling vessels lined by hypertrophied endothelial cells. One of the live healthy pheasants had a single small focus of gliosis and lymphoid perivascular cuffing in the cerebrum. The brain lesions were suggestive of a viral encephalitis and potential etiologies included avian influenza virus (AIV), avian paramyxovirus-1 (APMV-1), West Nile virus (WNV) and Eastern Equine Encephalitis virus (EEEV).

Following the identification of the brain lesions in both the dead and sick pheasants, immuno-histochemistry (IHC) for WNV and EEEV was conducted on formalin-fixed, paraffin-embedded brain tissue from the live sick pheasants. The results were positive for EEEV. Tracheal and pooled cecal tonsils from dead and live sick birds and brain tissue from two of the live sick pheasants were positive by rt-RT-PCR for EEEV thus confirming the IHC results.

This case is of interest because to the best of our knowledge, this is the first report of EEEV infection in pheasants in Canada.

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

CONTROL OF FOWL CHOLERA MORTALITY IN A BROILER OPERATION

B. Remington[A], P. Blackall[B], and C. Turni[B]

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[B]Queensland Alliance for Agriculture and Food Innovation, University of Queensland, St Lucia, Brisbane, Australia

SUMMARY

Fowl cholera was diagnosed on a multi-aged, organic, chicken meat farm which was suffering high mortalities. Control of the disease was achieved with a bacterin administered at day old. Subsequent vaccination failures were found to be a result of stress and concurrent disease. Only one genotype of *Pasteurella multocida* was found with that genotype being different to that of feral animals from the same farm. *P. multocida* was found to persist on the farm at least 12 months after clinical problems ceased.

INTRODUCTION

Fowl cholera is rarely diagnosed in broiler type chickens. Indeed, the disease is typically described as occurring in laying flocks with the additional comment that chickens less than 16 weeks age are quite resistant (1). In this report, we describe a case of a long term, high mortality situation in organic broiler chickens where the diagnosis was found to be fowl cholera. The therapy and preventative actions available in organic chicken production are limited if organic certification is to be maintained. A further complication was that the affected property contained a hatchery, processing plant, fertilizer composting facilities, and growing facilities. The individual growing sheds were operated on an all- in all- out basis. However, chickens had access to open ranges, the farm was multi-age, and biosecurity was poor. This report describes how control of this fowl cholera was achieved.

DIAGNOSIS

History. The onset of the problem occurred 12 months before this investigation. Since the onset, both the incidence and severity of the problem had increased to the point where all flocks were involved and the mortality to processing was averaging 55%.

Clinical findings. High mortality outbreaks occurred as early as 30 days of age. Large numbers - averaging over 3% per day - of sudden mortality were commonly seen. Birds in good condition were found depressed, then comatose and rapidly progressed to death. A few days into the course of the condition, small, depressed, sick birds appeared in large numbers. Cannibalism became a problem; however, it was noted that the disease appeared before the cannibalism emerged. Feral animal (cat and fox) predation was often observed before outbreaks.

Lesions. Principle gross lesions were hydropericardium, multiple white necrotic foci in the liver, and cardiac muscle hemorrhages. These hemorrhages are described as petechia on the internal thoracic pleura, within the coronary grove, and just under the visceral pericardium.

Presumptive diagnosis. On the basis of the clinical signs and lesions, a presumptive diagnosis of peracute or acute fowl cholera was made.

Confirmation of diagnosis. Aseptic swabs from pericardial sac, heart blood, liver, and bone marrow resulted in cultures of pure suspect *Pasteurella multocida*. Multiple single colonies were taken from the primary isolation plate of the bone marrow as well as single colonies from all sites and the resultant cultures were confirmed as *P. multocida* by a species specific PCR (2). All colonies were examined by an ERIC-PCR method (3) and all were shown to be a single genotype.

CONTROL

Biosecurity. Considerable effort was made to institute conventional biosecurity procedures to restrict the introduction of infection to flocks. This was pursued to the limit of the facilities available and the organic certification requirements. Successful action was taken to reduce the activities of native and feral animals. However no significant improvement in mortality result was gained for the effort.

Vaccination. A bacterin that used aluminium hydroxide gel as the adjuvant was prepared. The most practical method of vaccination was subcutaneous at day of hatch. Trials were conducted for 1) *safety* (100 bird treatment groups grown to seven days of age), which found no significant performance difference due to vaccination; 2) *protection of a single dose given at day old* (2,000 bird treatment groups mixed into each flock of 20,000 birds) resulting in a 65% protection; and 3) *vaccination of 100% of the flock at day old*, which gave 100% protection from all clinical fowl cholera.

Vaccination failures. At six months after the initial success of vaccination, 30% mortality due to fowl cholera was found in one flock. This flock was severely cold stressed at a day old. It was rationalized that the cold stress had depressed flock immunity. However, further outbreaks of fowl cholera occurred at a rate of one flock per month with 10%-30% mortality. The clinical findings and lesions were similar to the previous outbreaks. The genotype of *P. multocida* found in the vaccination failure cases was the same as that in the initial mortality cases diagnosed. Therefore the vaccine should still have provided protection. Another observation was the occurrence of poor gut health prior to an outbreak; severe enteritis was noted in the days immediately prior to an outbreak. Changes in coccidiosis vaccination resulted in marked improvement in gut health and no further fowl cholera outbreaks.

Subsequent findings. To determine the source of the infection, both feral cats and foxes trapped on the farm were sampled. While finding that they carried *P. multocida*, it was found that it was never the same genotype as that found in the chickens. Also, PCR of a suspect liver of a chicken with bacterial infection was positive for *P. multocida* 12 months after the last clinical outbreak.

DISCUSSION

This case demonstrated that fowl cholera can significantly affect young broiler chickens. While there

are reports that show an age influence (with increasing age, increasing susceptibility) (1), this case shows that under certain circumstances, broiler chickens as young as 30 days can suffer acute fowl cholera.

Once an infection with *P. multocida* has reached significant challenge levels it is difficult to practically remove it. It probably remains on farm and is not reintroduced. A similar conclusion has been reached in a study that showed fowl cholera outbreaks on a free range layer farm that were separated by eight years in time were due to the same genotype (4).

This work showed that one subcutaneous dose of an aluminium hydroxide gel adjuvant vaccine at day old does not significantly affect the production performance of a broiler chick. Further, one dose of a killed autogenous vaccine at day old provided protection against fowl cholera for the life of a broiler chicken.

As the genotypes of *P. multocida* found in the wildlife did not match the type associated with chickens, predation is probably not a source of infection but acts as a stressor. Other stressors – cold and coccidiosis – also had a role to play in apparent "vaccine failures."

We could find only one genotype of *P. multocida* to be responsible for the outbreak of fowl cholera initially examined, and all subsequent outbreaks on a farm.

This single genotype was found even through multiple tissues and multiple birds examined. This same finding of a single genotype has been reported in outbreaks in free range layers (4). Hence, apparent "vaccination failure" is not always caused by a change in challenge strain. It is worth noting that changes in genotypes across outbreaks have been reported in Denmark in a Muscovy duck operation (5).

Overall, there is a clear need for continued health monitoring and investigation to adapt disease control programs.

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ASSESSING THE IMPACT OF DIFFERENT HAND SANITIZING PROTOCOLS FOR CATCHING CREW MEMBERS

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SUMMARY

By handling animals and farm equipment, hands are exposed to pathogens. Many products are available to sanitize hands: nonantimicrobial and antimicrobial soaps used with water, waterless alcohol-based hand rubs, and waterless hand wipes. In human medicine, hand rubbing with a waterless alcohol-based product is the accepted standard for hand hygiene because it is microbiologically effective *in vivo* and *in vitro*, is easy to use, saves time, and improves hand hygiene compliance (Trampuz and Widmer, 2004). But these studies were performed on hands that were not visibly soiled. In the poultry industry, soap and water hand washing can be inconvenient since sinks are not often available in barns. There is only one study that tested alcohol-based hand gels as an alternative to soap and water after handling animals. Davis (2005) did not find any difference between treatments.

In the turkey and poultry industries, catching crew members can have heavily contaminated hands and could act as mechanical vectors for infectious diseases. Thus, it is important to reduce the microbial load on hands by performing effective hand sanitation.

No study has investigated the effectiveness of hand hygiene techniques in this context. The objectives were to evaluate the efficacy of practical hand sanitation methods and to determine the most practical approach according to users.

Enrollment of participants. This was an experimental study conducted under field conditions, performed between July and October 2010. A catching crew was followed during normal working hours and four volunteers were selected based on their willingness to participate. All catching crew members of the same company also received the products that were tested in order to try each of them at work in order to provide their opinion about the proposed protocols and products via a survey.

Hand sanitizing protocols investigated.

Protocol #1 (G): use of a waterless alcohol gel with 62% ethanol (Purell; Johnson & Johnson, Markham, Canada)

Protocol #2 (WaSG): use of water and antibacterial hand soap containing triclosan (Dial Spring Water; Henkel Consumer Goods Inc., Oakville, ON) and drying with paper followed by the use of the same waterless alcohol gel.

Protocol #3 (DCG): use of a degreasing pumice-added cream without water (Motomaster Heavy-duty Hand Cleaner with pumice; Motomaster Canada, Toronto, Canada) and wiping with paper followed by the use of the same waterless alcohol gel.

Protocol #4 (WG): use of detergent and antimicrobial wipes (Big Wipes; Sycamore Israel, Petach Tikva, Israel) followed by the use of the same waterless alcohol gel.

The time used by each person for each protocol was recorded using a video camera and the quantity of alcohol-based gel used was estimated by recording the number of times each participant pressed on the delivery device fixed on the waterless alcohol container.

Data collection. The four participants were asked to clean their hands with one of the four protocols studied in the project after catching birds. Samples were taken on their hands before and after applying each hand sanitizing protocol. Each protocol was applied eight times by each participant. They did not use the same protocol twice in a row (i.e., on a given work day, if they participated in loading three flocks of birds, they used three different protocols, starting with the fourth protocol on the next working day).

Sampling material and method. We used cloths soaked with a neutralizing broth *(D/E Neutralizing Broth, Lab M, Bury, Lancs; Roberts D. (1995))* contained in sterile sampling bags . The product was certified sterile by Labplas Inc., Ste-Julie, Quebec, Canada. The sampling material was kept at 4°C and transported in the field in a cooler with ice packs. After collection, the samples were transported back to the laboratory in another cooler with icepacks.

Prior to the application of any protocol, participants were asked to rub both hands together to reduce any potential difference in contamination between the right and left hands. A pre-treatment sample was taken on one hand by applying the sampling cloth on the inside of the hand and rubbing it with five circular motions going from the center of the hand to the periphery. Following this sampling, each participant sanitized his hands according to one of the four protocols. A second sample (post-treatment) was then taken on the other hand (the one not selected for pre-treatment sampling) using the same method as for the first sample.

Microbiological processing. All samples were processed at the Faculty of Veterinary Medicine the next morning following collection (within 12 h of collection). Ten mL of an enrichment media (buffered peptone water) were added to the sampling bags which were stomached for 30 sec in order to obtain a homogeneous sampling solution. The solution was serially diluted and spread on Aerobic and E.Coli/Coliform 3M Petrifilms (3M, St Paul, MN, US) in order to count total aerobic, E. Coli and coliforms population according to the MFHPB-33 and MFHPB-34 procedures of Health Canada. Then 100 mL were added to the sampling bags for the pre-enrichment for Salmonellas. The bags were then incubated at 37°C for 18 to 24 h. After that, the solution was spread on MRSV plates in order to detect the presence of salmonella according to the MFLP 75 procedure of Health Canada.

RESULTS

Salmonella **neutralization**. Only a limited number of dirty hands tested positive for Salmonella. All hands treated with any of the four protocols were subsequently negative for Salmonella.

Aerobics and coliforms. We used a linear mixed model with the number of bacteria on cleaned hands as dependent variable. The "protocol" (G, WaSG, DCG, or WG) was a fixed factor. The number of bacteria on dirty hands was a cofactor and the participant was a random factor (in order to consider repeated measures for each participant). We indentified a significant interaction between the variable « protocol » and the number of total aerobic bacteria on dirty hands, and a marginal interaction between « protocol » and total coliforms on dirty hands. This suggests that the efficacy of protocols varied depending on the initial hand contamination. Therefore, contamination on cleaned hands was analyzed depending on the level of initial contamination (before washing), i.e., each protocol was considered at three levels of

contamination: first quartile (lower 25% level of contamination), median (50%), and third quartile (level of contamination corresponding to the upper 25% of contamination). Variables such as the amount of disinfecting gel and duration of rubbing hands were controlled in the analysis. We also performed contrast assessments between protocols, depending on the degree of hand contamination. The Bonferroni sequential correlation procedure was used in order to take into consideration the number of comparisons between protocols.

Total aerobic bacterial count. There was no significant difference between protocols when initial hand contamination was low. However, the average contamination of clean hands was less for the WaSG protocol compared to the WG protocol when initial hand contamination was median and high.

Total coliforms. There was no significant difference between protocols when initial hand contamination was low or median. For high contamination, the average clean hand contamination was more elevated for the disinfecting gel alone versus the degreasing cream and gel protocol.

Based on the results, when hand contamination is low, all protocols work well. However, when contamination is higher, soap and water followed by a disinfecting gel seemed better to control aerobic bacterial count. Using a degreasing waterless cream followed by a disinfecting gel was better against coliforms. Clearly, all protocols had some value (although wipes had marginal value), and the difference between protocols was not as important as we expected. However, there is merit in using either soap and water or a degreasing cream when hand contamination is elevated. In places where water access is an issue, using a waterless degreasing cream would be valuable prior to using a disinfecting gel. However, if given a choice, the majority of the 38 catchers who responded to our survey preferred soap and warm water to the degreasing cream. Finally, it is interesting to note that the amount of disinfecting gel and the time spent rubbing it did not have a significant impact on decontamination. In other words, using the basic amount delivered by pressing the dispensing device once was sufficient.

(A full-length article will be submitted for publication in *Avian Diseases*.)

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DIAGNOSING METABOLIC ACID-BASE WET LITTER PROBLEMS USING A PORTABLE BLOOD GAS ANALYZER ISTAT-1

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SUMMARY

A case of wet litter in a broiler breeder flock was diagnosed using an iSTAT-1 portable blood gas analyzer. Metabolic alkalosis, high HCO_3^- and high base excess oriented the diagnosis towards a salt excess in the feed. Rapid changing of the feed allowed a rapid return to normal consumption of water and feed as well as a normal rate of lay. Feed analysis results one wk later confirmed the salt excess. The use of in-field

biochemistry can reduce the time required to diagnose and implement treatment in the case of wet litter problems and at the same time contribute to animal well-being.

Recent advances in technologies to analyze blood gases and electrolytes have permitted units to become portable or even hand held. Until recently these type of analysis were seldom used outside of research trials in poultry medicine because of poor practicality and high cost. Samples had to be transported and analyzed rapidly (within 20 min) for accurate results and the transportation of live birds to the laboratory could alter respiratory parameters because of transport conditions or delays.

This presentation will describe a clinical use of in-field portable blood gas analyzer iSTAT-1 for the rapid diagnosis of a wet litter problem associated with a drop in egg production in broiler breeders.

MATERIALS AND METHODS

Samples. Five birds were randomly selected for blood sampling. Venous blood was pulled from the wing vein using a one cc tuberculin syringe with a 25G x 5/8" needle. Samples were immediately dispensed into CG8+ or EC8+ cartridges according to manufacturer's recommendations.

Biochemical analysis. Biochemical analysis was done on a i-STAT-1 handheld Clinical analyzer (Heska Corporation, Loveland, CO, Abbott Point of care inc., East Windsor, NJ) using the CG8+ or EC8+ cartridges.

Feed analysis. Feed analysis was done on a pooled 500 g sub-sample of four samples taken in the feed bins at the time of the visit. The feed sample was analyzed at the Shur-Gain laboratory, St-Hyacinthe, Qc according to the following reference methods: sodium, potassium, calcium, phosphorous, magnesium MA#8, protein MA#2/Ma#20, chloride AOAC 969.10

Data gathering. Data for production and water consumption were gathered from different logs on the farm and biochemical results were compiled using an Excel spreadsheet. The results were compared to our database for interpretation in comparison with normal values for the type of production and the age.

RESULTS

Preliminary observations showed a severe problem with wet litter, feed refusal, increased water consumption, and a drop of seven percent in lay. No increase in mortality was noted. Birds were euthanized for on-farm macroscopic autopsy. No particular lesions were noted in the gut of birds except for excess fluid in the terminal part of the intestines.

Biochemical results at the time of the visit are presented in Table 1. Interpretation of the results, considering the venous origin of the blood, indicated elevated base excess associated with elevated bicarbonate levels. Birds with no respiratory acidosis tended to have lower than normal hematocrit values, which indicated water retention. This observation plays a role in the compensation observed after the treatment.

A decision was made to change the feed and empty left over feed in the feeders considering the metabolic aspect of the disorder and the uniformity of biochemical results of the sampled birds. Water was eliminated as a cause considering that the six other barns on the complex having the same water did not show clinical signs. Addition of chloride was not considered because of the elevated chloride level in tested bird.

Water consumption returned to normal the next day and feed consumption time came back to previous length. Rate of lay came back up five percent during the wk after the feed change. Blood samples analysis was done the next wk showing normal metabolic parameters but a severe respiratory acidosis as shown in Table 2.

The respiratory acidosis can be associated with pulmonary congestion and lower gas exchange at the lung level caused by the high salt levels.

One wk later, results of feed analysis showed that sodium level was at 0.39% corresponding to two fold the guaranteed label level. Chloride levels were also elevated (0.43%).

CONCLUSION

The use of in-field blood gas and electrolyte measurement is a practical and cost efficient tool that allowed a rapid evaluation of treatment options for this flock following a salt excess in the feed.

Table 1. Biochemical results at the time of the visit.

vBlood pH	7.3 -7.45 Database (7.30-7.35)	7.386	7.452	7.402	7.336	7.409
$vpCO_2$	40 -65	60.8	49.6	49.9	64	55.7
$vHCO_3^-$	25-33	36.5	34.6	31.1	34.3	35.3
vBeecf	-3 to +3	11	11	6	8	11
Ca^{++}	1.35- 1.55	1.68	1.57	1.61	1.54	
Cl^-	110-116					116
Na^+	135 -151	160	153	156	155	154
Ht	7.3 -7.45	27	23	23	29	28
Interpretation		Metabolic alkalosis	Metabolic alkalosis	Metabolic alkalosis	Metabolic akaliosis with respiratory acidosis	Metabolic alkalosis

Table 2. Blood samples analysis done the next wk showing normal metabolic parameters but a severe respiratory acidosis.

	Normal	Bird 1	Bird 2	Bird 3	Bird 4
vBlood pH	7.3 -7.45 Database (7.30-7.35)	7.144	7.067	7.133	7.215
$vpCO_2$	40 -65	75.7	100.1	88.2	75.7
$vHCO_3^-$	25-33	26	28.8	29.5	28.8
vBeecf	-3 to +3	-3	-1	0	1
Ca^{++}	1.35- 1.55	1.43	1.6	1.55	1.54
Na^+	135 -151	154	153	156	155
Ht	25-31	27	29	28	26
Interpretation		Respiratory acidosis	Respiratory acidosis	Respiratory acidosis	Respiratory acidosis

ISOLATION OF *MYCOPLASMA IOWAE* IN A COMMERCIAL TURKEY FLOCK – CLINICAL AND MICROBIOLOGICAL ASPECTS

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ABSTRACT

Mycoplasma iowae (MI) is an avian mycoplasma. Even though the turkey is considered the natural host, it has been isolated from different avian species. Clinical signs include decrease of hatchability and mortality in the late phase of egg incubation. Some authors reported poor growth and leg abnormalities. In the Italian poultry industry the presence of MI dates back to 1980.

In this study we report different clinical cases that occurred in 2011 in meat turkey flocks, where signs of increase of leg fracture and reluctance to move were associated to MI isolation in affected birds.

INTRODUCTION

MI is considered an important pathogen for the poultry industry, in particular for the turkey sector. The related disease in turkeys and chickens has been largely described under experimental conditions. The most important clinical finding is embryo death in turkeys and chickens. The microorganism was easily demonstrated in the intestinal tract of dead embryos. In turkey and chicken chicks it can cause a mild airsacculitis and increase of leg abnormalities. The last evidence of MI in commercial turkey was reported in the US in 2010 (2); whereas there are lack of recent evidence of MI infection in Europe.

In this study we described three clinical cases associated to MI isolation in commercial turkey flocks.

CASE REPORT

This case report deals with three commercial flocks of toms bred under standard commercial conditions. Flocks one, two, and three contained 20,560, 10,800, and 8,500 toms respectively. The vaccination programs applied in the farms are those routinely applied in commercial turkey flocks.

Flock owners reported a mortality rate of 3%, 1.5% and 2.5% respectively. Several carcasses belonging to the three different flocks were sent to the Diagnostic Unit of Istituto Zooprofilattico Sperimentale delle Venezie and submitted for necropsy examination. All examined groups showed a common history of mild respiratory form which appeared during the second to fourth wk of age. Moreover, flock one and two showed in the same period a mild intestinal disorder. Later, birds showed debility and leg weakness with an incidence of about 10% in flocks one and three and 30-40% in flock two. A smaller number of animals were unable to stand up and only strong mechanical stimulus obliged them to move. This problem was detected in flock one at 43 d, in flock two at 28 d, and finally in the flock three at 42 d.

A therapy with the association of calcium, vitamin D_3, biotin, and tylosin was applied in flock one and three, whereas in flock two a combination of doxycycline and gentamycin was administered instead of tylosin. The effect of therapy was partially successful and birds treated with gentamycin recovered a little bit better.

At the end of the clinical period the percentage of culled birds was 3-4% in flock one and three, and 18% (1,900 birds) in flock two.

MATERIALS AND METHODS

Ten birds from each flock were sent to the Diagnostic Unit of Istituto Zooprofilattico Sperimentale delle Venezie in order to perform Gross-pathology examination and other related diagnostic analysis. In particular, a transmission of electron microscopy from gut specimens and a specific PCR for *Astrovirus* was performed. Specimens of intestine, spleen, air sacs, and brain were submitted for routine microbiological tests under aerobic, microaerophilic, and anaerobic conditions. Finally, tracheal, gut, and cloacal samples were submitted for *Mycoplasma* spp. isolation based on our internal procedure.

RESULTS AND DISCUSSION

In all birds examined, the gross pathologic findings included poor feathering and marked soiled vents. The inspection of the musculoskeletal system showed abnormal leg development with intra-rotation of the metatarsus. The leg bones were rubbery and easily breakable. Moreover the upper part of the beak was soft and easily deformable. Very mild airsacculitis was found in some animals whose intestinal loops presented a watery contents and signs of enteritis. Parasitological examination of gut showed the presence of coccidia in flock one and a co-infestation of coccidia and *Trichomonas* spp. in flock two. The examination of the ceca provided standard results.

No evidence of enteric viruses was detected with the methods applied, whereas *Escherichia coli* was detected in all the examinated intestine and *Clostridium perfringens* was detected only in flock two; moreover, *E. coli* was also found in air sac specimens. Mycoplasma cultivation showed suspect colonies in all cloacal samples and in some specimens collected from the small intestine and trachea. The suspect colonies were identified as MI by immunofluorescence (IFAT) and DGGE method (3), confirmed by sequencing of 16S gene (1).

This report highlights the comeback of MI in the commercial turkey sector in Europe and it is one of the few reports describing a natural infection of this rare mycoplasma species.

The clinical signs and the gross pathologic findings are in accordance with those described under experimental and natural infections, enforcing its pathogenic role not only in the embryos but also in the growing phase of turkeys.

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CURRENT TRENDS IN TURKEY HEALTH, VACCINATION PRACTICES, AND WELFARE

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TURKEY HEALTH

Each year since 2005, the subcommittee chairman for the USAHA Committee on the Transmissible Diseases of Poultry and Other Avian Species, in conjunction with turkey industry colleagues, have surveyed turkey industry professionals and veterinarians representing a majority of the US turkey production regarding the health status of turkeys produced (2,3,4,6,7,8,9). Table 1 summarizes these results from 2005 to 2011. As can be seen from this table, the diseases/issues of most economic concern, which consistently appear include: lack of approved, efficacious drugs; clostridial dermatitis (cellulitis); colibacillosis; late mortality; and leg problems. On first look, the conclusion might be drawn that as an industry we are not attempting to make improvements. This is not the case however, as environmental management and biosecurity programs overall have improved, resulting in better performance and less mortality; however, the economic impact of these diseases has escalated over the years due to significant increases in feed and fuel.

Lack of approved, efficacious drugs. In 1987, dimetridazole was banned in the USA, leaving no approved drug for the treatment and prevention of histomoniasis. Although this disease may not rank as high as others, on average 80 cases have occurred annually for the past five years (range 63 to 108). Currently there are no effective treatments available worldwide for blackhead, which is of concern to valuable breeding stock in addition to commercial turkeys. The withdrawal of the NADA for enrofloxacin in 2005 for use in poultry leaves the industry with no adequate therapeutic response to colibacillosis or fowl cholera. At this time, we are also at risk of losing the ability to use injectable cephalosporins, which is the treatment of choice for cholera in breeding stock.

Clostridial dermatitis. Clostridial dermatitis, previously referred to as cellulites, has increased in ranking from 3.2 in 2005 to 3.9 in 2011. The disease was first observed in Minnesota in 1992 (1) and is now present in all geographic areas of the USA, affecting breeders and commercials, hens and toms. An increase in the severity of cases (scored 4–5) from 2007 to 2011, was reported by respondents from 26 % to 71 % . Mild cases (scored 1-2) decreased from 22% in 2007 to 12% in 2011. A Gold Medal Panel held in Dec. 2008 brought both researchers and industry together to discuss risk factors and potential causes (5). At present, treatment is through penicillin, and prevention through autogenous vaccines and biosecurity measures. Research on the pathogenesis and more effective control is on-going as infected farms continue to repeatedly break.

Colibacillosis. The ranking of *E. coli* has changed very little since 2005 (3.1 to 3.6) with the exception of 2006 when it rose to 4.0. This may be due to the ban of enrofloxacin in 2005. The turkey industry is continually striving to prevent primary stressors from occurring which allow secondary E. coli to cause disease. The continual ranking of colibacillosis in the top three diseases of economic importance is likely also the result of the frequency with which *E. coli* affects flocks at various ages. A specific survey in 2006 indicated that *E. coli* occurred in 18.4 % of flocks 0 to 5 wk of age, 19.8 % of flocks six to 12 wk of age and 10.7% of flocks greater than 12 wk of age (8).

Late mortality and leg problems. Late mortality may be defined as mortality in excess of 1.5 percent

per wk in toms (males) 17 wk and older; mortality is not diagnosed to a specific disease or cause. Excess cumulative mortality of five to ten percent in toms prior to slaughter has been reported. Late mortality may be associated with physiologic or biomechanical deficiencies following early rapid growth in heavy toms achieving genetic potential, aggressive behaviour noted in mature toms, cannibalism, leg problems, and/or hypertension (2). Since 2005, the ranking of this condition has show a steady decline from 3.5 to 2.8 in 2011, which is partly the result of improved selection pressure from the primary breeders for durability and delayed maturity, feeding to meet the nutritional requirements of the changing genetics, and improving litter conditions to reduce footpad dermatitis.

Leg problems are a common complaint, such as spiral fractures of the tibia or femur. Leg problems may be defined as lameness, particularly in toms, several wk prior to slaughter. Leg problems are attributed to various conditions, including pododermatitis, fractured femurs, fractured tibia, osteomyelitis (OM), tibial dyschondroplasia (TDC), spondylolisthesis, and "shaky leg." As late mortality decreased in ranking, so has leg problems, from a high of 3.3 to the current level of 2.8, for similar reasons.

Emerging diseases or those of concern. In 2011, a new disease agent emerged in the Midwest USA causing tenosynovitis and digital flexor tendon rupture. The causative agent has been identified as a reovirus, which is unique from types found in chickens or those involved in enteritis in turkeys. Affected flocks experienced lameness and abnormal gaits which lead to an increase in late mortality, and in some cases, an increased incidence of aortic ruptures and overall poor flock performance (weight gain, uniformity). In 2011, this disease was added to the turkey health survey and ranked #11 with 106 "confirmed" cases or flocks (2). One respondent noted that their operation processed over 300 flocks with varying degrees of severity. Research is on-going into pathogenesis, virus characterization, diagnostics, and epidemiology.

At one time, turkey breeding operations would immediately destroy any flock found positive for *Mycoplasma synoviae* (MS). The trend now however, is to keep these flocks in production and manage the disease through egg injection or dipping and/or treatment of poults at hatch and/or medication of commercial progeny during critical times. This change is likely the result of one or more of the following factors:

1. The disease risk or pressure of positive broiler breeders within the geographic area. Many broiler breeder companies have chosen not to depopulate and have elected to control the MS through vaccination programs.
2. Spread of MS through a large number of turkey breeder flocks such that if all positive flocks were destroyed the company would not be able to supply poults, which ultimately would result in empty shackles at the processing plant.
3. Some strains of MS that have resulted in few, if any, clinical signs under commercial conditions.

Vaccination practices. In 2005 H3N2 (swine influenza) affected many turkey breeder flocks resulting in significant egg production drops, which is reflected in the ranking of this disease as 2.9 in the health survey (9). The development of effective vaccines (autogenous and commercial) and increased biosecurity has decreased the economic impact of this virus to its current ranking of 2.0. However, this has come at a cost to the breeder industry because companies in high risk areas with close proximity to swine may need to vaccinate three times prior to egg production, and two to three times during production. Companies utilizing off site males have also had to vaccinate them and test frequently to ensure that influenza virus is not being shed and transmitted through semen to multiple hen flocks.

FSIS's focus on preharvest control of *Salmonella* has caused more companies to incorporate *Salmonella* vaccines (autogenous and commercial) as part of a strategic control program in breeders. Food safety recalls and the focus, especially on specific serotypes or ones that have multiple antibiotic resistance patterns, will also increase the use of *Salmonella* vaccines in the future in turkey breeders.

Turkey welfare. Animal welfare audits (internal, second, and third party) and training programs are now an integral part of every turkey company. These audits now encompass all segments, from breeders, hatcheries, farm, slaughter plant, and transportation and handling. Companies have invested considerable resources to ensure that all employees are trained and competent, especially in the areas of bird handling and euthanasia. Just as there is zero tolerance for biosecurity breaches, there is zero tolerance in most companies for acts of animal abuse. Viable alternatives for on-farm euthanasia of turkeys need to be identified, evaluated, and approved by organizations such as the AVMA. More research needs to be done to evaluate the effects of various management practices on the welfare and well being of turkeys. Areas of concern going forth include beak trimming on breeder hen replacements, molting of turkey hens, the establishment of mortality standards, and gait scoring. There is a gap between the animal production/welfare "experts", and production managers and veterinarians as to what exactly constitutes improved animal welfare. For example, some animal welfare scientists are promoting the idea that the highest level of welfare for turkeys is those raised free range with mobile slaughter

units coming to the farm. Many of these suggested practices will be at the expense of food safety.

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Table 1. Summary of turkey health surveys of US veterinarians in turkey production ranking current disease issues (1= no issue to 5 = severe problem).

ISSUE	2011	2010	2009	2008	2007	2006	2005
Lack of approved, efficacious drugs	4.3	4.6	4.7	4.5	4.7	4.4	4.2
Clostridial dermatitis / cellulitis	3.0	4.0	3.8	3.3	3.1	3.5	3.2
Colibacillosis	3.5	3.3	3.4	3.1	3.4	4.0	3.6
Late mortality	2.8	3.2	3.1	3.3	3.4	3.3	3.5
Leg problems	2.8	3.1	3.0	2.9	3.3	3.1	3.1
Heat stress	3.3	2.9	2.1	2.1	3.1	2.5	2.4
Poult enteritis of unknown etiologies	3.1	2.9	3.3	3.0	3.0	3.2	2.9
Bordetella avium	2.6	2.7	2.7	2.8	2.7	2.7	2.6
Breast blisters and breast buttons	2.6	2.6	2.6	2.4	2.7	2.4	2.4
Salmonella	3.1	2.6	2.5	2.6	2.7	2.8	2.8
Blackhead	2.2	2.5	2.5	2.3	2.1	2.1	2.1
Ornithobacterium rhinotracheale	2.4	2.4	2.5	2.3	2.4	2.8	3.0
Cholera	2.1	2.4	2.5	2.6	2.7	2.5	2.5
H3N2 swine influenza	2.0	2.0	2.3	2.3	2.4	2.4	2.9
Histomoniasis (Blackhead)	2.2	2.5	2.5	2.3	2.2	2.1	2.1
Turkey reovirus digital flexor tendon rupture (TR-DFTR)	2.5	0	0	0	0	0	0

CHARACTERIZATION OF REOVIRUSES ISOLATED FROM TENDONS OF TURKEYS PRESENTING WITH TENOSYNOVITIS AND DIGITAL FLEXOR TENDON RUPTURE

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SUMMARY

Avian reoviruses have directly or indirectly been associated with a number of disease conditions in chickens, including viral arthritis/tenosynovitis, enteritis/enteropathy (runting stunting syndrome), stunted growth, and poor feed conversion not caused by enteritis. Liver necrosis, perihepatitis, myocarditis, pericarditis, cardiomyopathy, hydropericardium, and depletion of the lymphoid cells in the spleen, thymus and bursa of Fabricius are also observed individually or as a disease complex following exposure to selected reoviruses.

In turkeys, reoviruses have been shown to affect the cardiovascular system, deplete lymphoid tissues, and induce an enteritis similar to that seen in chickens. However, reoviruses have rarely been reported as the cause of arthritis and ruptured tendons in commercially produced turkeys. Recently (2009-2011) a syndrome initially affecting primarily tom turkeys raised in the upper and lower Midwest was observed by multiple producers. The condition was characterized by an overt lameness and swollen hocks and shanks usually in turkeys older than 14 wk. On necropsy, the most common lesions observed were ruptured digital flexor tendons and synovitis. Reovirus antibody titers as assessed by commercially available ELISAs appeared to be elevated in affected flocks although reovirus antibody was also common in unaffected flocks.

Frozen whole legs were submitted to AviServe LLC from affected flocks for virus isolation attempts. The highest rate of reovirus isolations was obtained from turkeys ranging in age from 37 to 56 d that were lame but did not, in several cases, display signs/lesions typically associated with viral arthritis/tenosynovitis. A total of four reoviruses were isolated from digital flexor tendons, synovial fluids, or gastrocnemius tendons collected from turkeys produced in Missouri, Arkansas, and Minnesota. In several cases, reoviruses were also isolated from intestinal tracts of turkeys with viral arthritis. Interestingly the intestinal isolates differed antigenically from the tendon isolates and were not capable of inducing viral arthritis when inoculated via the foot pad into one d old turkeys.

Antisera was prepared against one of the reovirus isolates and utilized in microtiter neutralization assays to compare the antigenic configuration of each of the four isolates. Based on this assay, all of the viruses appeared to be the same serotype. However, the viruses were very poorly neutralized by antisera prepared against chicken viral arthritis virus (S1133). Even though the turkey isolates differed antigenically from S1133, they were pathogenic for chickens when introduced via the foot pad. This means that conventional chicken viral arthritis vaccines will not protect chickens against infection with the turkey isolates.

An *in vivo* comparison of the antigenicity and pathogenicity of the four turkey reovirus isolates was done in susceptible turkeys. Based on cross protection studies, the viruses appeared to be antigenically the same although there were modest differences in relative pathogenicity with the Minnesota isolates being relatively more virulent. One of the Minnesota reovirus isolates was inoculated into one d old turkeys by the foot pad or oral/intratracheal routes. When birds were sacrificed and necropsied at 14 wk, both of the inoculated groups presented with flexor and gastrocnemius tendon ruptures and synovitis similar to what was seen in naturally affected turkeys. Reovirus was also re-isolated from several of the affected birds.

Different approaches to control are being evaluated, including immunization of parent flocks with autogenous vaccines prepared from one of the characterized Minnesota reovirus isolates. In addition, a chicken reovirus isolated from seven d old broilers, shown to be antigenically similar to the turkey isolates but apathogenic for turkeys, is being assessed as a potential live vaccine. Results of the aforementioned strategies will be discussed.

FIELD OBSERVATIONS OF TENOSYNOVITIS AND DIGITAL FLEXOR TENDON RUPTURE IN CENTRAL USA TURKEY FLOCKS

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SUMMARY

A condition characterized by unusual lameness and aortic rupture was observed in tom turkeys over 14 wk of age located in Southwest Missouri. Occurrences began in December, 2010 and progressed for nine months before subsiding in the fall of 2011. Affected flocks first exhibited bilateral lameness described as a slow, painful, unsteady gait with the absence of limping. It was also noticed that the lateral toes on one or both feet were extended so that they rarely made contact with the floor. Flocks eventually increased in mortality due to apparent aortic rupture. A more thorough necropsy of the mortality revealed a significant correlation of aortic rupture and digital flexor tendon rupture. Significant thickening of the pericardial sac and loss of cardiac muscle tone were also noticed.

After discussion with other turkey veterinarians who had identified the same condition, a decision was made to attempt isolation of reovirus from the impacted tendons. Efforts failed when older, chronic cases were sampled but were successful from younger, acute cases.

Flock performance history revealed that the condition results in a mortality increase of 3% to 10%, depending on severity. Market weights, feed efficiency, and DOAs at the processing plant were also significantly impacted. Histories also indicated no lateral transmission of the virus and the infection is not repeated on subsequent flocks in the same confinement buildings. However, evidence does suggest the condition repeats in flocks originating from the same breeder flock.

INVESTIGATION AND CONTROL OF TURKEY TENOSYNOVITIS AND DIGITAL TENDON RUPTURE

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SUMMARY

In late 2009 and early 2010, a lameness and swollen hock joint condition in 15 wk old and older tom turkeys was observed in numerous different turkey production sites throughout the upper Midwest area of the United States. Affected turkeys were reported in Minnesota, Iowa, North Dakota, and South Dakota. Later in 2010, male flocks in Missouri and Arkansas were also observed with some of the same presenting conditions. As this condition progressed within a flock, many turkeys would go down. This resulted in heavy culling and lead to high mortality due to the inability of birds to get to feed and water and to avoid interaction with more aggressive birds in the flock. Severity of the condition varied from flock to flock, with mortality between 2% and 35%. The number of birds affected in a flock also varied and could be as low as 2% and as high as 70%. Both hen and tom flocks were affected, but hens were generally marketed before clinical signs were noticed. When affected turkeys were examined, the lesion did not appear to originate from the hock. A rupture of one or more of the digital flexor tendons and a synovitis condition which appeared to be a chronic

inflammation was noted. This suggested that the condition started early in the bird's life.

A group of turkey veterinarians from the areas impacted was formed into a task force along with Dr. John K. Rosenberger of AviServe. Ideas were shared which lead to the direction of the investigation into this condition. Several studies were completed in a very short time. These included: characterization of four reovirus isolates from the lesions of turkey flocks suffering from viral arthritis; comparative pathogenicity of the four reovirus isolates; reproduction of the condition in 13 wk of age tom turkeys that were foot pad injected as day old poults; reproduction of the condition in 13 wk of age tom turkeys that were orally intra-tracheal inoculated; reisolation of the virus from the tendon/hock joint of those 13 wk old birds; cross neutralization assay; protection of turkey poults to vaccination with chicken reovirus vaccine; and neutralization and protection of the four isolates.

One isolate was then selected and made available and developed into a killed vaccine. This autogenous vaccine is currently being used by a large breeder-hatchery company in an attempt to control turkey viral arthritis. Progeny from these vaccinated breeder flocks are at this time still in the field but results will be available by this paper's presentation.

AN OVERVIEW OF ANTIMICROBIAL USE AND ANTIMICROBIAL RESISTANCE IN CANADIAN TURKEYS

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ABSTRACT

In 2009, the turkey meat per capita consumption in Canada was 4.4. kg/person, making turkey the fourth most commonly consumed meat in Canada. While chickens have been extensively investigated for antimicrobial resistance (AMR) characteristics, little is known about AMR in Canadian turkeys. More recently in the United States, the consumption of turkeys contaminated with multi-drug resistant (MDR) *Salmonella* enterica serovar Heidelberg has been associated with human outbreaks in multiple states. In light of the potential human health impact of the consumption of turkey meats contaminated with resistant organisms, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) initiated surveillance of ground turkey meats at retail level in 2011. This program monitors trends and patterns of antimicrobial resistance in generic *Escherichia coli*, *Salmonella*, and *Campylobacter*. It is methodologically similar to the United States National Antimicrobial Resistance Monitoring System (NARMS). This paper provides an overview of current antimicrobial use (AMU) practices in the Canadian turkey industry, and prevalence and antimicrobial resistance of enteric bacteria isolated from turkey meats.

INTRODUCTION

The turkey industry in Canada operates under a supply management system, i.e., the industry is controlled by the Turkey Farmers of Canada (TCF) in a manner similar to other poultry commodities (e.g., hatching eggs and chicks, broilers, table eggs). In 2009, total turkey meat production was approximately 165.5 million kgs, placing Canada as the eighth leading turkey producer worldwide (3); and per capita consumption was 4.4. kg/person, making turkey a major source of animal protein for Canadians (1). Antimicrobial-free and organic turkey production systems have become popular in the last decade; however, national production estimates are unavailable, so the proportion of turkeys are raised under these production systems is unknown.

As with the top three major food animal species in Canada (chicken, cattle, and pigs), enhanced biosecurity, on-farm food safety quality assurance programs, and industry-wide vaccination are used to prevent disease. In the face of an outbreak, veterinarians and producers may use prophylactic or therapeutic antimicrobials to reduce the economic and animal welfare impact of bacterial diseases; however, the emergence of AMR in turkeys poses a public health concern. In 2011, a human salmonellosis outbreak in the US was attributed to consumption of turkey meat contaminated with *Salmonella* Heidelberg resistant to ampicillin, streptomycin, tetracycline, and gentamicin (6). No similar outbreaks have been reported in Canada.

CIPARS, a national program operated by the Public Health Agency of Canada in conjunction with government, industry, and academic partners, monitors AMR in various food animal commodities. Since 2002, CIPARS has collected AMR information in turkeys through the passive surveillance of *Salmonella* isolates obtained from clinical cases submitted by veterinarians and/or producers to the Canadian

diagnostic laboratory network. In 2008, *Salmonella* isolates exhibited high levels of resistance to several antimicrobials. These antimicrobials included: ceftiofur (56%), tetracycline (44%), gentamicin (28%), and streptomycin (41%); resistance to trimethoprim-sulfamethoxazole was relatively low (3%) (2). Aside from the passive surveillance of clinical *Salmonella* isolates from turkeys, there has been no national program that monitors AMR in clinically-relevant organisms in Canadian turkeys (e.g., *Pasteurella, Staphylococcus, Bordetella, Erysipelothrix*). To assess the potential public health risk of turkey consumption, a study examining the prevalence and AMR of bacteria isolated from turkey meat was conducted in Ontario in 2003-04 (4), and in Alberta in 2007-08 (5). Retail food samples best reflect consumer exposure to resistant food-borne bacteria. In February 2011, CIPARS initiated surveillance of retail ground turkey to monitor AMR trends and patterns in *E. coli, Salmonella,* and *Campylobacter*, and to bring CIPARS into closer harmonization with the National Antimicrobial Resistance Monitoring System (NARMS) in the United States. This paper provides an overview of current antimicrobial use practices in turkey production in Canada, recovery rates of enteric pathogens, and associated AMR profiles.

MATERIALS AND METHODS

AMU. To describe antimicrobial therapy for common turkey pathogens, literature searches of *in vivo* (e.g., animal/clinical trials, pharmacokinetic-pharmacodynamic studies) and *in vitro* (e.g., based on antimicrobial susceptibility testing of isolates) were conducted from January to December 2011 using electronic databases and poultry-specific online journals, such as Avian Diseases and Poultry Science Journal. All studies including those conducted internationally from 1966 to 2011 were assessed. Antimicrobials were grouped according to their importance to human medicine, using the categorization system of Health Canada's Veterinary Drugs Directorate (VDD) as follows: Category I - Very High Importance; Category II - High Importance; Category III - Medium Importance, and Category IV - Low Importance (7). For review of available guidelines, the Canadian Association of Veterinary Medicine Prudent Use Guidelines (CVMA-pug) 2008, the 2009 Compendium of Veterinary Products (CVP), and Compendium of Medicating Ingredient Brochure (CMIB) online were reviewed to determine applicable guidelines and policies regarding veterinary antimicrobial use in Canada.

AMR. Following CIPARS procedures for sampling retail meats, sampling of ground turkey meat involved regular sample collection from randomly selected census divisions, weighted by population, in each participating province/region (Maritimes, Québec, Ontario, Saskatchewan and British Columbia). Details regarding methods used for sample collection, culture, and antimicrobial susceptibility testing are available in the CIPARS annual reports (8).

RESULTS AND DISCUSSION

Antimicrobial drug use. Literature search yielded information on the antimicrobials presented in Table 1. Some antimicrobial drugs used for prophylactic and therapeutic purposes are (or belong to classes that are) essential for serious life-threatening infections in humans such, as ceftiofur and enrofloxacin. The antimicrobials listed in Table 1 are available in Canada, but, if used in turkeys, some of these would have to be used in an extra-label manner because they are not approved for one or more of the following:

1) use in turkeys.
2) the indications described (e.g., target pathogens and/or disease conditions).
3) route of administration described.
4) the dosage described.

Most of these drugs require prescription. Drugs that are for over-the-counter (OTC) purchase are relatively few and generally include the older antimicrobials including penicillin, streptomycin, and the tetracyclines. Valid veterinary-patient-client relationship (VPCR), prescription only medication, extra-label drug use considerations (ELDU), and compliance with the CMIB (e.g., in-feed use) are some of the prudent antimicrobial drug use practices intended to reduce public health hazards.

Bacterial recovery rates. (Note: bacterial recovery rates and AMR data are preliminary (Feb. 2011 – early Dec. 2011), final data for 2011 will be presented at the conference.) Preliminary recovery rates of bacteria from retail ground turkey in 2011 were: *E. coli* – 295 isolates/317 samples (93%), *Salmonella* – 49/359 (14%), and *Campylobacter* – 37/359 (10%). Of the *Salmonella* that were serotyped and phage typed to-date, the most common *Salmonella* serovars were: *S.* Agona 7/39 (18%), *S.* Enteritidis, *S.* Hadar, *S.* Heidelberg, *S.* Schwarzengrund (all 4/39 [10%]), followed by *S.* Albany 3/39 (8%). Of the 21 *Campylobacter* that were further characterized, six were *C.coli* and 15 were *C. jejuni*.

Antimicrobial resistance.

E. coli: Preliminary results are presented in Figure 1. The percentage of isolates resistant to ceftiofur and ceftriaxone was 12% (36/295). No resistance to ciprofloxacin was detected, however, 2% (6/295) of the isolates were resistant to nalidixic acid.

Relatively high prevalences of resistance to ampicillin, streptomycin, and tetracycline were observed (>30%).

Salmonella: Preliminary results are presented in Figure 2. Only 31 of the 49 isolates recovered were susceptibility-tested at the time that this short paper was submitted. The percentage of isolates resistant to ceftiofur and ceftriaxone was 29% (9/31). Relatively high prevalences of resistance to streptomycin, sulfisoxasole and tetracycline were observed (>19%).

Campylobacter: Of the 21 isolates that were susceptibility tested to-date, ten isolates were resistance to tetracycline. All isolates were susceptible to all other antimicrobials tested (azithromycin, clindamycin, erythromycin, gentamicin, nalidixic acid, and florfenicol).

CONCLUSIONS

Antimicrobials are available in Canada for the therapy of bacterial diseases in turkeys, but some of the uses described in the literature would have to be used in an ELDU manner with veterinary supervision. Resistance to various antimicrobials is present in enteric bacteria isolated from retail ground turkey meat. Prudent use practices are recommended to preserve the efficacy of these antimicrobials. CIPARS will continue to monitor AMR trends and patterns in retail ground turkey meats.

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Table 1. Literature review of antimicrobial[a] therapy of common turkey pathogens.

	E. coli	***Staphylo-coccus***	***Bordetella avium***	***Erysipelo-thrix rhusio-pathiae***	***Ornitho-bacterium rhinotracheale***	***Pasteurella multocida***	***Salmonella arizonae***
I[b]	*Ceftiofur,* Rx[c]			*Enrofloxa-cin,* Rx	*Enrofloxacin,* Rx	*Enrofloxacin,* Rx	
II	Gentamicin (SC only), Rx *Lincomycin-Spectinomycin* (Inj) *Ormetoprim-sulfadimethox-ine,* Rx *Trimethoprim-sulfadiazine,* Rx	Erythromycin *Ormetoprim-sulfadimethox-ine,* Rx Penicillin G *Trimethoprim-sulfadiazine,* Rx	*Ampicillin,* Rx *Gentamicin,* Rx *Ormetoprim-sulfadimethox-ine,* Rx Penicillin G *Trimethoprim-sulfadiazine,* Rx	Erythro-mycin Lincomycin Penicillin G	Erythromycin *Tilmicosin* Spectinomycin	Neomycin-Oxytetra-cycline *Ormetoprim-sulfadimetho xine,* Rx Penicillin G *Tilmicosin Trimetho-prim-sulfadiazine,* Rx	*Gentamicin,* Rx
III	Chlortetracycli ne *Sulfamethazine Sulfaquinoxa-line* Tetracycline		Chortetra-cycline Oxytetrac-ycline Tetracycline	Specino-mycin	*Florfenicol,* Rx	Chlortetra-cycline *Florfenicol,* Rx Oxytetra-cycline Tetracycline	Spectino-mycin Tetra-cycline
I V							

[a] Cited by various authors and are available in Canada for veterinary use. Italicized antimicrobials are used in an extra-label manner for indications, species, or dose.
[b] Roman numerals I to III indicate the ranking of antimicrobials based on importance in human medicine as outlined by the Veterinary Drugs Directorate, Health Canada
[c] Rx- prescription only recommended by manufacturer.

Figure 1. Resistance to antimicrobials in *E. coli* isolates from retail ground turkey meats.

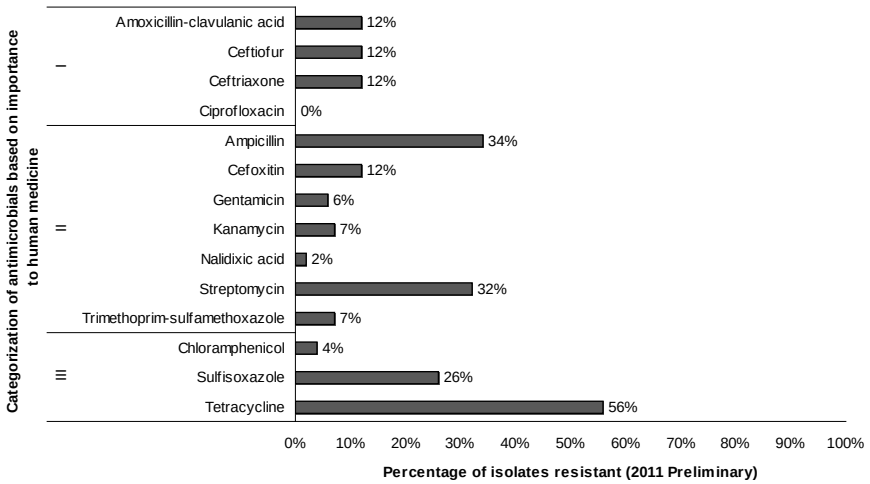
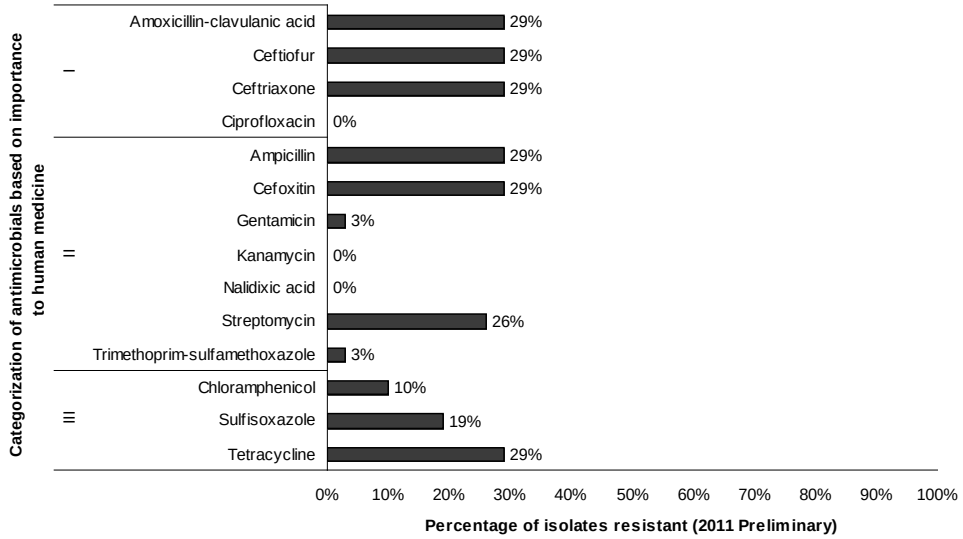


Figure 2. Resistance to antimicrobials in *Salmonella* isolates from retail ground turkey meats



A REVIEW OF IBD VIRUSES ISOLATED FROM US BROILERS FROM 2009-2011

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ABSTRACT

A total of 117 infectious bursal disease virus (IBDV) sequences were identified by our imaging/PCR lab from broiler samples between 2009 and 2011. Phylogenetic analysis of these 117 sequences showed that the vast majority fell into three large clades: AL2 types, G6 types, and Del-E types, in order of prevalence. While IBDVs from all three clades infected flocks before and after three wk of age, Del-E types almost exclusively infected prior to three wk.

INTRODUCTION

IBDV is a leading cause of immune suppression in broiler flocks due to its destruction of B-lymphocytes in the bursa of Fabricius and other peripheral lymphoid organs. Infections prior to three wk of age have the potential to cause the most profound and long-lasting immune suppression and are therefore the number one priority in the United States, where antigenic variants predominate the broiler landscape. The last major phylogenetic analysis of IBDVs was published in 2005 (1). This analysis showed the emergence of a clade (G6) that had eclipsed the Delaware viruses as the most prevalent in U.S. broilers. The purpose of this paper is to provide a more recent "snapshot" of IBDVs in U.S. broilers with the additional perspective of age of infection.

MATERIALS AND METHODS

Five to six bursas were collected from each broiler flock in the survey. Bursas were split lengthwise and their respective halves placed into either formalin containers for computer imaging analysis, or freezer bags for PCR analysis. After computer imaging results were generated, frozen bursa samples from suspect IBDV-positive flocks were submitted to The Ohio State University for PCR analysis (RT-PCR and sequencing analysis) (1, 2). Phylogenetic analysis was performed using the Lasergene 8 software package from DNA Star.

RESULTS

One hundred seventeen sequences were recovered from 114 farms (three dual infections) from nine different companies and a total of 33 different broiler complexes. A total of 12 states (# isolations per) are

represented as follows: AL (31), AR (23), GA (14), DE (9), MS and NC (8), IN and VA (7), CA and MO (4), and KY and TN (1). The phylogenetic tree (see Figure 1) divides the IBDVs into three general branches or clades: AL2 types (46%), G6 types (30%), and Del-E types (20%), with just a handful of IBDVs falling outside these branches.

The single largest AL2 clade consists of 54 IBDVs. Of these, 28 are identical to the AL2 prototype (with mutations 318-N and 321-E in hydrophilic Peak B). Two other large subgroups in the AL2 clade differ from AL2 by having either 318-S or 321-D. The second largest G6 Clade, named after Jackwood's original terminology for this family of viruses, contains the most diversity of the three general branches. The two most common G6 virus types, Cal-6 (named after Jackwood's 1174) and T1 (3), make up 27 of the 35 IBDVs in this group. Lastly, the Delaware Clade consists of 23 total IBDVs. Of these, nine are identical to Del-E, while another nine Del-E viruses had one to two additional mutations in 254-N and/or 299-S.

Time of infection was also estimated for each isolate using imaging analysis (4). The goal was to see if any of the aforementioned virus types tended to be recovered preferentially from late infections or early ones (<21 d of age). In fact, two virus types did show a propensity to infect more often in one of the two infection windows. Del-E types, including those containing one to two additional mutations in 254-N and/or 299-S, were most often recovered from early infections. In contrast, AL2 viruses, but not AL-like viruses, were more often recovered from late infections. A summary of these results can be seen in the provided Table 1.

DISCUSSION

While it is clear that there continues to be a shift in the IBDVs in U.S. broiler flocks away from Delaware type viruses to New-types, such as G6 and especially AL2, the age and frequency of these infections is also important to consider. The fact is, most U.S. broiler flocks today are protected from IBDV before three wk of age. For example, we sampled over 500 flocks between 11-32 d of age to yield the 114 IBDV positive flocks. Using imaging analysis, we estimated that all IBDV positive flocks were infected between 14-27 d of age, with 82 isolates infecting before 21 d of age, and the remaining 35 infecting later.

An examination of the 18 Del-E type viruses shows that while they only made up 15.4% of all 117 viruses, they were still quite prevalent in flocks infected before three wk of age. For example, 22.0% (18/82) of all early infections were caused by Del-E types compared to only 2.9% (1/35) of all late infections. So even in the presence of Del-E focused maternal immunity, this virus still appears to compete well against other New-types in young flocks (but not in older ones).

Another unexpected finding was that a comparison of the 28 AL2 viruses showed the opposite trend. That is, AL2 viruses identical to the original prototype virus were recovered from 37.1% (13/35) of all late infections compared to only 18.3% (15/82) of early infections. Numerous progeny challenge studies have demonstrated AL2's ability to infect two wk old commercial broiler flocks at a higher rate than Del-E (5). However, perhaps this survey data is not so much a statement about AL2's ability to infect broilers at an early age as it is a suggestion that it may compete even better against other IBDVs when the infection window is late.

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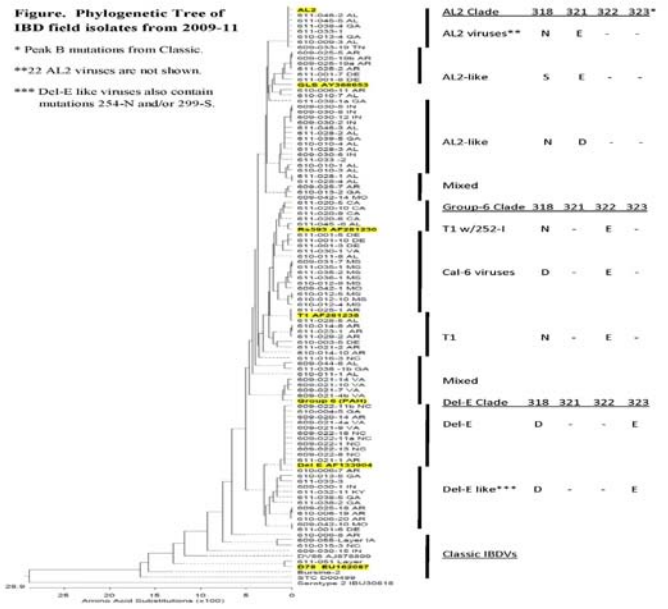
Table 1. Comparison of IBDV types by age of infection.

IBDV type	Infections <21 d		Infections ≥21 d		P-value*
	number	percentage	number	percentage	
AL2	15	18.3	13	37.1	0.1278
AL2-likes	15	18.3	6	17.1	1.0000
Cal-6 types	10	12.2	5	14.3	0.9960
Del-E types**	18	22.0	1	2.9	0.0545
T1 types	7	8.5	3	8.6	1.0000
Mixed	17	20.7	7	20.0	1.0000
Total	82	100.0	35	100.0	

* Statistical analysis using Fisher's exact test with multiple comparisons controlled for by permutation resampling.
** Del-E types include viruses containing additional mutation of 254-N and/or 299-S.

Figure 1. Phylogenetic tree of IBD field isolates – 2009-11.



MOLECULAR ASSESSMENT OF A VECTOR HVT-IBDV VACCINE (VAXXITEK HVT+IBD®) INTAKE AND REPLICATION

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SUMMARY

This work evaluates vaccine intake and replication after hatchery application of a commercial vHVT-IBDV vaccine (VAXXITEK HVT+IBD®) using a specifically designed qPCR assay. Feathers from vHVT-IBDV vaccinates were tested for vaccine virus at different time points. A second trial tested blood, buffy coats, bursa, spleen, and FTA® card samples between four and 18 d. A third trial assessed the vaccine replication in free range chickens up to 81 d old. Results showed between 60% and 100% positive detection in feather tips (maximum expression at d 28). Vaccine replication can be consistently detected in

spleen (fresh or FTA cards), fresh buffy coats, or bursas from d 11. Free range vaccinates were 98% and 88% positive at 35 d and 81 d respectively. These results indicate the presence of vaccine virus during the bird's infectious bursal disease (IBD) susceptibility period and demonstrated the suitability of fresh feathers and spleen qPCR detection for vaccination quality control assessment.

Marek's disease (MD) control by vaccination in the broiler industry is successfully achieved by using turkey herpes virus (HVT), and/or SB1 and Rispens MD commercially available vaccines (3). IBD control in broilers is attempted using live and/or killed vaccines for the breeders and progeny with variable success (5). More recently, i*n ovo* or day old subcutaneous vaccination with HVT vectors expressing the IBDV viral protein 2 (VP2) (vHVT-IBDV) allows protection against both MD and IBD (variant and classic) in the presence of high maternal derived antibodies and represent the current trend in the poultry industry (1, 2, 5). However, due to the kinetics of the HVT vector vaccines in the flock (no horizontal transmission and no field revaccinations), assuring adequate vaccine intake by optimizing vaccination protocols is vital for vector vaccines success. To provide tools and enhance quality control (QC) in vector vaccination protocols, this work aims to evaluate the vaccine intake and replication after hatchery application of VAXXITEK HVT-IBD, which is a recombinant HVT expressing the VP2 from a IBDV classic strain (Faragher 52/70) by using a specific qPCR analysis of fresh tissue and FTA cards samples.

MATERIALS AND METHODS

A specifically designed quantitative real time polymerase chain reaction (qPCR) assay was used to assess vaccine intake and replication. In a first trial, feathers from vHVT-IBDV laboratory reared broilers and from field vaccinates (fresh and FTA cards) were tested for specific vHVT-IBDV at different time points. A second trial tested blood, buffy coat, bursa and spleen samples between four and 18 d and assessed the use of FTA cards for sample preservation. A third trial assessed vHVT-IBDV vaccine replication in brown chickens reared up to 81 d of age.

RESULTS AND DISCUSSION

In vector vaccine technology, no revaccination is recommended and the vector vaccine does not transmit horizontally, indicating that vaccine/vaccination QC is of foremost importance. Monitoring immunization and assuring positive vaccine virus replication in the bird is the only way to provide protection against the vector and the transgenic immunogenic protein. Results showed positive vHVT-IBDV detection between 60% and 100% in fresh feathers with maximum expression observed at 28 d, while inconsistent results were observed for FTA feather samples. Results are summarized in Table 1. The second trial demonstrated that the vaccine virus replication can be consistently detected in spleen (fresh or FTA cards), or from fresh buffy coats and bursas from d 11. Spleen samples are the most relevant material to gather, from D11 for both fresh tissues and FTA-transferred tissues. Replication of the vHVT-IBDV during the birds IBD susceptibility period is very important. In this trial, free-range vaccinates were 98% positive at 35 d and 88% at 81 d of age. These results are consistent with recent work demonstrating the suitability of vHVT-IBDV vaccines for longed lived commercial layers. Perozo et al. (2010) reported that layers between of four and 20 wk, vaccinated at d one with the vHVT-IBDV vaccine showed significantly higher spleen weight ($P<0.0001$), bursa weight ($P<0.0001$), bursa diameter ($P<0.001$), bursal /body weight ratio ($P<0.001$), and bursal /spleen ratio ($P<0.001$). These results correlated to lower histopathological bursal lesions, suggesting a better immune status when compared with a live vaccine program. In conclusion, these results indicate the presence of vaccine virus during the bird's IBD susceptibility period and demonstrated the suitability of fresh feathers and spleen qPCR detection for vaccination QC assessment.

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Table1. qPCR vHVT-IBDV detection in broilers vaccinated at one d and reared in the laboratory or the field from feather tips or FTA cards.

qPCR vHVT-IBDV detection	Unvaccinated				Laboratory/Vaccinated				Field/Vaccinated		
	D20	D35	D28	D42	D20	D35	D28	D42	D21	D28	D21 (FTA®)
Positive vHVT-IBDV samples	0/10	0/10	0/10	0/10	6/10	8/10	10/10	9/10	13/15	12/14	4/15
vHVT-IBDV gene copy number / 2 feathers	0	0	0	0	0 to 17	0 to 6,200	0.5 to 36,000	0 to 5,300	0 to 430,000	0 to 160.000	0 to 220
Beta actin	+	+	+	+	+	+	+	+	+	+	+
vHVT-IBDV positive control	+	+	+	+	+	+	+	+	+	+	+
vHVT-IBDV negative control	-	-	-	-	-	-	-	-	-	-	-

BUSINESS CONTINUITY FOR EGG PRODUCERS DURING AN HPAI OUTBREAK: THE FAST EGGS PLAN

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Goal and objectives. On April 1, 2010, the Federal and State Transport (FAST) of Eggs Plan became operational in Iowa. The goal of the Plan is to facilitate business continuity by allowing non-infected premises within an HPAI Control Area to obtain permits to move eggs and egg products into market channels after eggs are held in cold storage for 48 h. Objectives of the FAST Eggs Plan are as follows:

- Minimize the risk of exposure of poultry flocks to HPAI and thereby limit the spread of HPAI during an outbreak.
- Provide a high degree of confidence that whole shell eggs entering market channels for human consumption are free of HPAI virus.
- Reduce the time needed to meet regulatory criteria for moving whole shell eggs into market channels.

Biosecurity. Minimum biosecurity standards are preapproved by the State Veterinarian and federal Area Veterinarian in Charge (AVIC). The Iowa FAST Eggs Plan's "Biosecurity Checklist for Egg Production Premises and Auditors" contains 45 important biosecurity measures. These measures were developed by a panel of poultry veterinarians with expertise in egg production and avian influenza, State and Federal epidemiologists, egg producers, Iowa's State Veterinarian and federal AVIC, and the Center for Food Security and Public Health (CFSPH) at Iowa State University. Implementation of these biosecurity

measures prior to an outbreak will significantly reduce the likelihood that HPAI virus will be introduced onto egg production premises.

Registration and location verification of Iowa FAST Eggs Plan premises using global positioning system (GPS) coordinates. Participation in the Iowa FAST Eggs Plan is voluntary and premises-specific. Egg producers who choose to participate in the Iowa FAST Eggs Plan are required to register their premises with Dr. David Schmitt, the State Coordinator and State Veterinarian. Participants may opt to register their premises in the FAST Eggs Plan online or by mailing or faxing forms to the State Coordinator. A Trainer/Auditor employed by the State Coordinator collects farm identification and location data for each participating egg operation on Day 1 of participant training, including the farm longitude and latitude by using a GPS receiver (see G below).

Epidemiology questionnaire and flock data. In the event of an outbreak of HPAI, managers of participating egg operations will provide data by answering questions on an epidemiology questionnaire available through a premises-specific data portal. Foreign animal disease investigators will use this information to determine whether a FAST Eggs premises has been exposed directly or indirectly to birds and other animals, products, materials, people, or aerosol from an infected premises. At the start of an incident, in addition to the epidemiology questionnaire, participating facilities will be required to submit information on daily mortality and daily egg production for the preceding seven days for each chicken house on a premises via a premises-specific website. Participating premises managers will be required to report significant unexplained changes in feed consumption, water consumption, or behavior. This information will be electronically submitted directly to the Incident Commander (IC) each day via a data.

Active surveillance by real-time reverse transcriptase polymerase chain reaction (RRT-PCR) testing. The potential presence of H5 or H7 avian influenza virus infection on a FAST Eggs Plan premises during an outbreak will be monitored by requiring chickens from each house on the farm to be tested each day by the HPAI RRT-PCR test. Daily surveillance requires one RRT-PCR test for each pooled sample of five dead or euthanized sick chickens per 50 dead chickens from each house on the premises. A minimum of five dead chickens from daily mortality or from euthanized sick birds from each house (flock) must be tested each day. Two negative RRT-PCR pools on the first day of testing or two negative RRT-PCR pools on consecutive days are sufficient to allow eggs which have been stored on the premises for at least two days in cold temperatures to be moved into market channels for human consumption. On subsequent days, one pooled sample from each house on the premises must test negative by RRT-PCR for HPAI. Before movement permits for whole shell eggs will be issued, in addition to negative PCR tests, chickens in all houses on the premises must be free of clinical signs of disease, have no unexplained increase in mortality, and have no unexplained decline in egg production or feed.

Secure data portal. Data from the biosecurity checklist, audits, and GPS coordinates, are entered into a premises-specific database prior to an outbreak of HPAI using data collected on Day 1 of participant training. Responses to the epidemiology questionnaire, flock production data, and daily RRT-PCR test results are entered at the beginning of an outbreak. Information is stored in a database administered by the State Coordinator with support from Iowa State University's CFSPH. All registered egg producers have a unique login and password to access the data portal. In the event of an HPAI outbreak, egg producers must complete the online epidemiology questionnaire and enter their premises-specific flock production data. Each day during an HPAI outbreak, ICs will be able to access this information electronically.

Participant training and pre-positioned instructions (Day 1). A Trainer/Auditor travels to an egg farm and conducts a morning training session which includes watching an instructional DVD explaining how to collect oropharngeal swabs. Thereafter, farm personnel participate in a wet laboratory consisting of hands-on collection of oropharyngeal samples from dead chickens. At the conclusion of the training session, farm managers are given:

- An instructional DVD explaining purpose of the Iowa FAST Eggs Plan and procedures for collection of oropharyngeal swabs
- Plastic-coated summary sheets of the oropharngeal sample collection process which can be used at the site of sample collection
- A customized submission form for the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL)
- Driving directions to the ISU VDL.

Certificates listing the names of individuals who have successfully completed the training session and the training date are issued to the farm managers.

Practice specimen collection and submission (Day 2). Farm personnel trained on Day 1 collect oropharyngeal swabs from five dead chickens from each house on the premises and swirl the swabs in brain-heart infusion broth (BHI). One labeled BHI tube from each house must be submitted to the ISU VDL by noon on Day 2. RRT-PCR tests for avian

influenza are conducted during the afternoon and results are electronically reported to the State Veterinarian and federal area veterinarian in charge (AVIC, Dr. Kevin Petersburg) by the end of the day. Farm managers must record the time required to collect and deliver samples to the ISU VDL so this information is known prior to an outbreak of HPAI (or END).

Biosecurity audit. Minimum biosecurity standards must be in place before a premises is accepted into the Iowa FAST Eggs Plan. The Trainer/Auditor conducts a biosecurity audit of the premises and submits a report to the State Coordinator/State Veterinarian (Dr. David Schmitt) and the federal AVIC (Dr. Kevin Petersburg). Dr. Schmitt and Dr. Petersburg jointly decide if biosecurity on a premises meets minimum standards necessary for participation. Premises biosecurity audits may be conducted on Day 1 of participant training or at a later date.

Enrollment, pre-positioned supplies, and website training (Day 3). After a premises has been approved for participation in the Iowa FAST Eggs Plan by the State Coordinator, a website is created for the premises and the premises is assigned a participant number. Farm managers are given instructions for ordering Dacron swabs and BHI tubes. BHI tubes can be kept in the refrigerator for 18 months and the number of BHI tubes in storage should equal five times the number of houses on a premises. The Auditor/Trainer provides computer training which instructs farm managers on the process of accessing and entering epidemiology, mortality, and egg production data on the farm-specific website.

EVOLUTIONARY AND TRANSMISSION DYNAMICS OF REASSORTANT H5N1 INFLUENZA VIRUS IN INDONESIA

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SUMMARY

H5N1 highly pathogenic avian influenza (HPAI) viruses have seriously affected the Asian poultry industry since their recurrence in 2003. The viruses pose a threat of emergence of global pandemic influenza, through point mutation and/or reassortment leading to a possibility that a strain can effectively transmit among humans. Up until 2012, no novel HP H5N1 that can infect and transmit efficiently among human has emerged. In this study, we have studied and present phylogenetic evidence for reassortment among H5N1 HPAI viruses isolated between 2003-2007 from humans and birds in Indonesia, and identify the potential genetic parents of the reassorted genome segments. Parsimony analyses of viral phylogeography suggest that the reassortant viruses may have originated from Greater Jakarta and the surrounding area, and subsequently spread to other regions in the West Java province. In addition, Bayesian methods were used to elucidate the genetic diversity dynamics of the reassortant and non-reassortant parental viruses, which revealed a more rapid initial growth of genetic diversity in the reassortant viruses relative to the non-reassortant viruses. These results demonstrate that inter-lineage exchange of genetic information may play a pivotal role in determining viral genetic diversity in a focal population. Moreover, our study also revealed significantly stronger diversifying selection on the M1 and PB2 genes in the lineages proceeding, and subsequent to the emergence of, the reassortant viruses, respectively. We discuss how the corresponding mutations might drive the adaptation onward and transmission of the newly formed reassortant viruses as well as how infectious viral disease emerges in a new geographic region.

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MONTANIDE™ ADJUVANTS FOR MUCOSAL DELIVERY OF LIVE AVIAN VACCINES

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SUMMARY

Live vaccines are widely used in the avian industry and are usually not adjuvanted. Here we demonstrate the improvement of live vaccines by adjuvants in a model of mucosal delivery of live infectious bronchitis vaccine in chickens. Three adjuvant technologies were used as diluents for the lyophilized antigen. Day old chickens were vaccinated by either intranasal delivery or spray. Compared to a commercial non-adjuvanted reference, intranasal delivery of polymer and nanoparticles adjuvanted live vaccines improved significantly the antibody titers observed and both adjuvanted vaccines strongly reduced the clinical signs scoring after challenge. Moreover, spray delivery of polymer adjuvanted vaccine also showed a significantly higher protection compared to the non-adjuvanted reference. Our data demonstrates that mucosal vaccine delivery of adjuvanted live vaccine confers to vaccinated animals a significantly improved protection against pathogens.

INTRODUCTION

Live vaccines are widely employed in avian practice (3). Based on the infectious properties of the attenuated or genetically modified live micro-organism, these types of vaccines exist for bacterial (6), viral (12), and parasitic (5) pathogen models. The live pathogens delivered by vaccine inoculums are thought to initiate both humoral and cellular protective response after one delivery. However, up to 10% of vaccinated animals can present a lack of protection after live vaccine delivery in the field and may represent a reservoir for pathogens (3). Improvement of the safety and efficacy of live vaccines is therefore an important issue.

Several improvements are expected from the addition of adjuvants in live vaccine formulations. The most important would be a reduction of the antigenic dose delivered, which would lead to safety improvements, cost limitations, and a better control of risks linked to the vaccination procedure (11). The Montanide™ range of adjuvants is a well established brand of vaccine adjuvants (1) which is already used in all farm animals models at industrial scale in combination with diverse types of antigens. In this study we assessed the efficacy and safety of Montanide adjuvanted mucosal live infectious bronchitis (IB) vaccines in poultry.

IB virus is a coronavirus that affects airways, digestive system, kidneys and reproductive system in poultry (4). Commercial live vaccines against IB are widely used in chicken breeding. We chose this model to study the effect of three families of adjuvants on the improvement of an IB commercial live vaccine in chickens delivered by individual, intranasal, or collective spray methods. We could show that Montanide Gel 01 ST and Montanide IMS 1313 N VG adjuvanted formulations improved significantly the antibody titers and protection provided by intranasal delivery of live commercial IB vaccine, whereas only the polymer adjuvanted vaccines showed a significantly better efficacy compared to the commercial reference in the spray assay.

MATERIALS AND METHODS

Animals. One day old SPF chickens (Lohmann Tierzucht, Germany) were seronegative to infectious bronchitis virus at day zero. Ten animals were included in each vaccine and control group. All protocols were validated by internal ARRIAH's ethics committee prior to launch according to OIE recommendations.

Antigen. The antigen used was IB virus strain H-120 from SPF egg embryos (ARRIAH). Vaccine antigen titer was log 4.0 egg infective dose 50 (EID50) per vaccine dose. All adjuvanted test vaccines and the commercial non-adjuvanted positive control contained the same amount of antigen per dose delivered to chickens.

Adjuvants. Montanide IMS 1313 N VG (IMS 1313 N), Montanide ISA 201 VG (ISA 201), and Montanide Gel 01 ST (Gel 01) were used in this study. Montanide IMS is a ready to dilute range of adjuvants consisting of liquid particles (10-500 nm) dispersed in an aqueous phase containing an immunostimulating compound. Montanide ISA is a ready to use range of

oil adjuvants that can be used to manufacture different types of emulsions. ISA 201 allows the formulation of water-in-oil-in-water vaccines. Montanide Gel 01 is a ready to dilute polymeric adjuvant. It contains gel particles of sodium polyacrylate in water.

Vaccination and experimental groups. All adjuvants were formulated extemporary to vaccination. A commercial live IB vaccine (strain H-120) produced by FGI 'ARRIAH', Russia (batch 211, control 211, use-by 09.2009) was used as a positive control; this vaccine does not contain any adjuvant. Ninety chickens were randomly separated into nine groups of ten chickens. In groups one to four, animals were vaccinated by intranasal delivery (IN) with the corresponding group test vaccine (i.e. IMS 1313 N adjuvanted vaccine, ISA 201 adjuvanted vaccine, Gel 01 adjuvanted vaccine or commercial non-adjuvanted control). Each animal received one antigen dose in 0.1 mL injected evenly in both nostrils. In groups five to eight, animals were vaccinated by the spray method with the corresponding group vaccine (i.e. IMS 1313 N adjuvanted vaccine, ISA 201 adjuvanted vaccine, Gel 01 adjuvanted vaccine or commercial non-adjuvanted control). For each group, 2.5 cm^3 of solution containing ten doses of vaccine were sprayed over ten animals in a box. Spray procedure was performed upon a high volume but the average of antigen dose received by the ten vaccinated chickens was identical to the individual IN vaccination procedure dose. Group nine (negative control) was left unvaccinated.

Safety. Animal behavior was followed before and after vaccine delivery in order to identify any modification related to the vaccination procedure.

Serology. Blood samplings were performed at d 0, 7, 14, 21, 28, 35, 42, 49 and 56. Antigen specific antibodies were detected individually at each date by antigen specific ELISA (IB antibody detection kit: ProFLOK® IBV ELISA, Synbiotics, Lyon, France).

Challenge. An infectious challenge procedure was performed at d 56 by delivery of 0.1mL in each nostril of a highly virulent IB virus strain (M-41; titer: log 5.0 EID_{50} per mL). After challenge procedure, animals were observed over ten d post infection for IB specific clinical signs presence.

Statistics. Statistical analysis was performed using Student's t tests and proportion tests. Results were considered as significantly different when $P \leq 0.05$.

RESULTS

General tolerance to the adjuvanted formulations. No modification of animal behavior (social, movements, feeding) could be observed after vaccine delivery in any of the protocols. Furthermore, no IB specific clinical signs could be observed after vaccine delivery. Lastly, no local reactions on the mucosa (eye, nostril, or mouth) to vaccine delivery could be observed after IN or spray vaccination.

Efficacy of spray and IN delivery of IB adjuvanted vaccine. Using IN delivery, Gel 01 and IMS 1313 N adjuvanted vaccines were able to trigger a significantly stronger humoral immune response than the non-adjuvanted commercial reference. IMS 1313 N group showed a faster response than the non-adjuvanted reference. Antibody titers of the ISA 201 vaccinated group were higher but not significantly different from the commercial control. After challenge, unvaccinated animals all showed clinical signs specific of IB infection as soon as six d post infection (Figure 1a). Gel 01 and IMS 1313 N adjuvanted vaccines conferred an improved protection to the vaccinated animals compared to the commercial reference (Figure 1a).

Spray delivery was not as efficient as IN delivery. Antibodies titers obtained after spray vaccination were lower than the IN induced titers for all groups. No adjuvanted group showed significant improvements compared to the commercial formulation. A faster onset of the response could still be observed (as soon as d 14 for adjuvanted vaccines, and d 21 for the commercial formulation). However, after challenge, the Montanide Gel 01 ST based formula could induce a high rate of protection when used in spray (88% of animals without symptoms at d ten post challenge, Figure 1b) and had a significantly higher efficacy than the commercial formulation.

DISCUSSION

Vaccination is one of the most powerful tools to improve farming efficiency and the protection of herds (7). However, it is not possible to add multiple injection vaccination steps in avian farming procedures due to the cost and time consumed by such steps in farms containing several thousands of animals. Mucosal spray vaccination could then be a very useful tool for time and cost efficient vaccination of large groups of animals (2).

We have shown previously that nanoparticles vaccine adjuvants can enhance the efficacy of avian mucosal vaccination against parasitic disease (8). In this study we have demonstrated that following individual intranasal delivery of adjuvanted formulations containing live viral vaccines, nanoparticles, or polymer based adjuvant technologies were able to improve the immune response and protection to IB challenge. Both Montanide Gel 01 ST and Montanide IMS 1313 N VG adjuvants conferred a significantly enhanced protection to challenge compared to the non-adjuvanted commercial reference. Montanide ISA 201 VG is a mineral oil adjuvant used for the formulation of water in oil in water emulsion

vaccines that can be used for injectable delivery in chicken (9), but we show in this study that nanoparticles and polymer adjuvants give much better results for mucosal delivery in poultry.

Using spray delivery, the polymer based adjuvant was the only adjuvant formulation able to confer protection at a very high rate and to be significantly more efficient than the commercial formulation. Spray vaccination is usually less efficient than intranasal delivery due to losses linked to non-specific mucosal delivery on the animals. Our results show that the use of a relevant adjuvant can allow mass spray delivery of live vaccines. Further studies should assess if the protective stimulation using spray vaccination that we observed using polymer adjuvanted vaccine was restricted to the nasal mucosa or was also linked to the oral and eye surfaces which would lead to different immune system / antigen contact (10).

Benefits anticipated from the use of adjuvants in live vaccines concern both safety and efficacy improvements. The use of adjuvants in live vaccine could improve the efficacy and lead to a better management of the antigen load per vaccine dose. Moreover, the use of adjuvants should reduce the number of low or not responding animals and therefore reduce the possible reservoir for the disease (3). Our work underlines the ability to use polymer adjuvants in mass vaccination for avian species, opening doors to improvements of live avian vaccines safety and efficacy.

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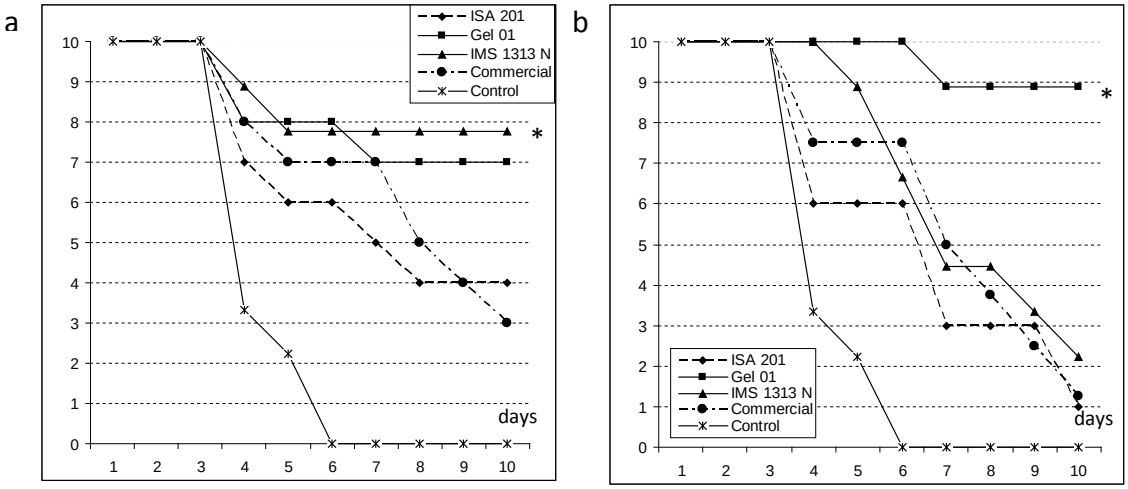
Figure 1: Efficacy of mucosal delivery of adjuvanted live IB vaccines on protection to challenge: animals without symptoms observed for ten d after challenge. (a) Intranasal delivery (b) Spray delivery. The control group is the non-vaccinated group.



* indicate significant differences compared to the commercial reference

TARGETED SURVEILLANCE FOR AVIAN INFLUENZA IN POULTRY IN IRAN IN 2009

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INTRODUCTION

Avian influenza (AI) is an infectious viral disease of poultry. The majority of AI infection in poultry is of low pathogenicity, causing mild to moderate clinical signs. Some AI viruses (subtypes H5 and H7) have the potential to mutate into highly pathogenic strains that can cause very high mortality in some poultry species. In 2008 Iran Veterinary Organization (IVO) introduced an annual serological and molecular survey with two objectives: firstly to detect subclinical infections with low-pathogenicity avian influenza (LPAI) of subtypes H5 and H7 to complement early detection systems, and secondly, demonstration of disease-free status of states, region, or compartment from notifiable avian influenza according to OIE regulations. The survey continues to improve knowledge on which sectors of the poultry industry (broiler breeder, laying breeder, or laying hens) and backyard (chicken, duck, geese, or turkeys) are more likely to be infected by H5 or H7 LPAI, and identified other sectors which are consistently more likely to be affected. Review of the result of surveillance, the global picture of avian influenza and scientific research in this field, will further improve AI future outbreaks, prevention, and control preparedness including the appropriate allocation of resources in this area.

THE SURVEY PROGRAM

The survey was conducted between October and December 2009 according to specific guidelines laid down in commission decision by Avian Influenza National Committee and financed by IVO. Four different geographical regions with a background of previous infection with low or highly pathogenicity AI were targeted to be sampled (Gilan in North, Tehran in Central, Fars in South, and Sistan in East). The survey design was based upon the surveillance guidelines. The required number of holdings to be sampled for specific poultry categories was determined according to tables as laid down in the guideline.

SAMPLING AND METHODS

Provincial Veterinary Services (PVS) were trained epidemiologically and laboratorialy. They sampled targeted holdings and submitted data for some or all of the following poultry categories: chicken breeders, laying hens, fattening turkey, ostrich, and backyard flock. Sampling was targeted towards backyard flocks (local chicken, ducks, geese, and turkeys) and commercial poultry with a long life span (breeders and laying hens). For backyards, five to ten per holding (ten holdings in each epidemiologic unit), and other categories, 25 serological samples were required to be collected from each farm to ensure a 95% probability of identifying at least one positive bird if the prevalence of seropositive birds was >30%. Additional sampling of 14 tracheal/cloacal swabs samples per 78 distinct holding in each seropositive village or farm for virological investigation which was tested as pools of five samples was carried out. Tracheal/cloacal swab from serologically positive holdings have been submitted to Central Veterinary Laboratory (CVL) for molecular test (RT-PCR). IVO is responsible for the collection of surveillance serological data which was done first by PVS. Data has been collected via paper reporting system operated by Department of Poultry Diseases Control. The required number of holdings to be sampled for specified poultry categories was determined according to the tables as laid down into the guideline. It was recommended that testing of samples should be carried out at provincial laboratories authorized by CVL. All AI virus isolates should have been submitted to the CVL. Viruses of H5/H7 subtype were subjected to the standard characterization test (nucleotide sequencing). Sera with HI titers of greater or equal to 2^4 were scored positive.

RESULTS

A total of 355 poultry holdings (13156 sera) for the following categories were sampled by the four PVC: broiler breeder (17 holdings), laying hens (42), laying pullet (two), backyard epidemiological unit (266 villages), and partridge (one). In Gilan, two broiler breeder holdings and 26 villages were sampled from Anzali region. In Tehran, 10 broiler breeder holdings, 34 laying hens, two laying pullet, and 59 villages from Savegbolagh region were sampled. In Fars, four broiler breeder holdings, six laying hen holdings, one ostrich holding, one partridge, and 91 villages were sampled from Shiraz region. In Sistan, one broiler breeder, two laying hens, and 90 villages from Zahidan region were sampled.

Fars. The required number of holdings was sampled for the chicken breeders, laying hens, and villages. Samples were taken from 91 villages including 4015 chicken, 211 turkey, 130 geese, 33 ducks, and 100 pigeons. One thousand five hundred eighty-seven chickens, 30 turkeys, ten geese, two ducks and 14 pigeons were serum positive for subtype H9, and 12 backyard chickens were positive for H5. No positive commercial holdings were reported, while samples were taken from 305 laying hens, 228 broiler breeders, 18 ostrich, and ten partridges. Unlike the previous years, H5 serologically positive villages' holding were reported.

Tehran. Samples were taken from 59 villages including 1984 chicken, 132 turkey, 62 geese, 71 ducks, and 200 pigeons. Five hundred sixty-six chickens, 19 turkeys, one duck, and 22 pigeons were serum positive for subtype H9, and nine backyard chickens and one pigeon were positive for H5. Three chickens were serologically positive for H7. Forty-six chickens, three turkeys and three pigeons were serologically positive for avian influenza A viruses, but none were H5, H7, or H9 subtype. Forty-six laying hen pullets, 1355 laying hens, and 324 broiler breeders were sampled. Thirty laying hens and one broiler breeder were H5 serum positive and one of laying hen sample was positive for H7.

Sistan. Two thousand one hundred ninety-seven chickens, 322 turkeys, eight geese, 13 ducks, and ten pigeons were sampled from 90 villages around Zahidan in south east of Iran. One thousand five hundred sixty-six chickens have been detected as H9 serum positive. Ten chickens were H5 positive. 68 turkeys were H9 serum positive. Thirteen chicken and seven turkey samples were other subtype, neither H5, H7 nor H9. No positive samples were reported in laying hen and broiler breeder.

Gilan. Nine hundred fifty-one chickens, 21 turkeys, 31 geese and 189 ducks were sampled from 26 villages in Anzali near to Caspian Sea. Twenty-eight chickens were serologically positive for H5, 11 for H7 and 67 for H9 subtype. Two geese were serologically positive for H5, one H7 and one H9 subtype. Two ducks were H5 positive and four H9 positive. No positive samples were reported in broiler breeder.

In this year's survey, two states found H7 subtype positive holding for the first time since 2004. In total, positive samples have been found in all four states. Forty nine holdings tested positive for H5 in four states and 14 subtypes H7 in two states. The results of the 2009 AI survey for poultry showed considerable variation amongst states. However, one feature is that all states that included backyard in their surveillance detected positive holdings in this sector. In two provinces, backyard chickens were the only type poultry detected with H5 AI. Although, the total number of local chicken accounted for 85% of the overall number of backyard holding tested, almost 96% of H5 AI infections were detected in this poultry

sector. Half of the serologically positive backyards were found in Gilan. Positives in ducks and geese (0.74%) were attributed to H5 subtype, with less to H7 subtype (0.36%) and small numbers of unspecified influenza A viruses were detected by Gilan, Tehran, and Sistan using a prescreening test for influenza A. However, a prescreening test was not conducted in breeder and layer hen due to H9N2 mass vaccination, so on the basis of these findings it cannot be assessed whether the proportion of H5 positive holdings compared to the proportion of positives for other subtypes. In two states, AI was detected in more backyard poultry than in the previous year. The proportion of positive holdings amongst the sampled in this year's survey increased in the majority of poultry categories and states compared to the previous year. Although the survey design in Iran has been changed, this survey could be an indication that there is obvious increase of an overall H5 prevalence. However it should be kept in mind that the sample numbers calculated for this survey were calculated with the objective to detect infection if it were to be present and not to estimate the prevalence and due to this statement must be taken with care.

Attempts to isolate viruses from serologically positive flocks should be maximized since there was not any virus isolation in this survey. All of the above information is very important for further improvement of control strategies for AI and to establish an evidence-based prioritization of resources for surveillance and control measures such as vaccination.

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RECOMBINANT VIRUS-VECTORED LARYNGOTRACHEITIS VIRUS VACCINES: EVALUATION OF EFFECTIVENESS BASED ON POST CHALLENGE VIRUS REPLICATION IN VACCINATED BROILER CHICKENS

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SUMMARY

Effectiveness of two recombinant virus-vectored laryngotracheitis virus (LT) vaccines was evaluated for prevention of LT in broiler chickens: a herpesvirus of turkeys-vectored [rHVT-LT] vaccine (CEVA Vectormune-HVT LT) and a fowl poxvirus-vectored [rFPV-LT] vaccine (CEVA Vectormune-FP LT).

Protective immunity produced by *in ovo* vaccination with rHVT-LT and rFPV-LT was compared with immunity produced by conventional vaccination with modified-live, chicken embryo-origin (CEO) vaccine administered in drinking water. Protective immunity in vaccinated chickens was assessed based on clinical signs, mortality, microscopic tracheal lesions, and quantitative detection of virus (infectious virus and viral DNA) in tracheal exudates following challenge with virulent LT virus. Assessment of protective immunity based on post challenge clinical parameters correlated well with quantification of infectious virus, but not quantification of viral DNA. Based on post challenge assessment of clinical parameters and quantitative detection of infectious virus in tracheas, chickens vaccinated with rHVT-LT or rFPV-LT had protective immunity that was comparable to immunity induced by CEO vaccine.

Chickens vaccinated with rHVT-LT, rFPV-LT, or CEO vaccine had low clinical scores, no mortality, and less severe tracheal damage (microscopic lesion scores) after LT virus challenge, compared with nonvaccinated chickens. Additionally, chickens vaccinated with rHVT-LT, rFPV-LT or CEO vaccine had reduced duration of detectable infectious virus and significantly reduced ($p < 0.05$) concentrations of infectious virus within tracheal exudates compared with nonvaccinated chickens. Infectious LT virus titers in vaccinated chickens (rHVT-LT, rFPV-LT, CEO) post challenge were not significantly different ($p < 0.05$).

Quantification of viral DNA (genome copies) in tracheal exudates using a real-time PCR procedure indicated reduced duration and reduced concentrations of viral DNA in vaccinated chickens (rHVT, rFPV-LT, CEO) compared with nonvaccinated chickens. However, reductions in viral DNA in tracheal exudates of chickens vaccinated with rHVT-LT and rFPV-LT were not significantly different compared with unvaccinated chickens, and viral DNA frequently was detected in tracheal exudates in the absence of detectable infectious virus.

The findings indicate effectiveness of *in ovo* administered rHVT-LT and rFPV-LT vaccines for prevention of disease in broiler chickens and indicate that effectiveness is comparable to conventional vaccination with CEO vaccine. Quantitation of infectious virus in tracheal exudates collected post challenge from chickens vaccinated with rHVT-LT and rFPV-LT indicate that these vaccines induce protective immune responses within the trachea that impede LT virus replication. The findings also indicate that quantification of infectious virus rather than viral DNA should be the standard for assessment of vaccinal immunity produced by LT vaccines. Real-time PCR detected presence of LT virus DNA in tracheal exudates in the absence of detectable infectious virus.

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

DETECTION AND DIFFERENTIATION OF AVIAN METAPNEUMOVIRUS BY REAL TIME RT-PCR

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SUMMARY

Direct diagnosis of avian metapneumovirus (AMPV) infections relies on molecular techniques more than on virus isolation due to the fastidious nature of the virus. Six real-time reverse transcription PCR (RRT-PCR) protocols for the detection and differentiation of AMPV subtype A and B were developed in N (two tests), F, SH (two tests) and G genes. Five assays used SYBR Green I as detection system, and one used molecular beacon probes. Specificity was evaluated using various AMPV strains, Newcastle disease, infectious laryngotracheitis, and infectious bronchitis viruses. All tests were able to detect AMPVs and failed to detect non-AMPV viruses, and five out of six of them were also able to discriminate between AMPV A and B subtypes. Sensitivity was determined using serial dilutions of RNA from AMPV of both subtypes. The best results in terms of specificity and sensitivity was given by the RRT-PCR protocol designed in the SH gene.

INTRODUCTION

Avian metapneumovirus (AMPV) causes an upper respiratory tract infection in turkeys and in some other avian species, including chickens. In laying or breeding birds the virus can cause drop in egg production and poor eggshell quality. These conditions are associated with serious economic losses in unprotected birds especially when secondary pathogens become involved (6). AMPV belongs to the genus *Metapneumovirus* in the subfamily *Pneumovirinae* of the family *Paramixoviridae*. The genome contains eight genes in the following order starting from 3': nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), matrix 2 (M2), small hydrophobic (SH), attachment glycoprotein (G), and large polymerase (L). Based on nucleotide sequence differences four subtypes of AMPV (A, B, C and D) have been identified. AMPV subtypes A and B are widespread in Europe (6).

Direct diagnosis of AMPV infections relies on molecular techniques more than on virus isolation due to the fastidious nature of the virus.

Consequently several RT-PCR, and more recently real-time PCR, protocols based on different genes have been developed and successfully used for detection and differentiation between AMPV subtypes (5, 7).

In the present study, attempts were made to develop a real-time PCR assay able to detect and differentiate AMPV subtype A and B. Different sets of primers and two different fluoregenic chemistries were used. A selected molecular beacon RRT-PCR was compared to an established subtype A and B specific RT-nested PCR (10), using serial dilutions of titered virus suspensions and RNA extracted from swabs collected after experimental infections.

MATERIALS AND METHODS

SYBR® Green I RRT-PCR

Primer design. Four sets of primers, based on different genes of AMPV (N, F, SH and G), were designed with the aid of the Beacon Designer 7.1™ software. An additional set of primers, first reported by Bäyon-Auboyer *et al.* (1) and based on the N protein gene, was evaluated in comparison.

Virus strains and RNA extraction. The following AMPV strains were used to assess the SYBR Green I RRT-PCR protocols: IT/Ty/Vr240/87 and IT/Ty/129-18/04 (subtype B) and IT/Ty/A/259-01/03 (subtype A). The strains of AMPV of subtype B were isolated in Italy in different years and belong to different genetic clusters (4) while the strain AMPV of subtype A was isolated in Italy in 2003 (9). IT/Ty/Vr240/87 (AMPV-B) and IT/259-01/06 (AMPV-A) had a titer of $10^{5,47}$ $TCID_{50}$/mL and $10^{5,25}$ $TCID_{50}$/mL respectively. RNA extraction was performed using the QIAamp® Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions.

SYBR Green I one-step RRT-PCR

SYBR Green I one-step RRT-PCR was performed using a LightCycler® 480 (Roche, Basel, Switzerland). The final reaction mixture volume of 20 µL contained 0.4 µL of SuperscriptIIIRT/Platinum® Taq mix, 10 µL of 2X SYBR Green Reaction Mix, 0,8 µL of each primer (10 µM) (forward and reverse), 6,8 µL of RNase-free water and 2 µL of template RNA. All samples were run in duplicate. Thermocycling parameters were as follows: 50°C, three min (reverse transcription); 95°C, five min (RT inactivation and activation of Taq DNA polymerase); 40 cycles combining 95°C, 10 s (denaturation) and 60°C 30 s (annealing, extension step and fluorescence data collection). Upon completion of the amplification, the specificity of the amplified product was confirmed by using melting curve analysis whereby the reaction mix was firstly incubated at 40°C for 45 s and then the temperature was increased rapidly to 95°C at a rate of 0.11 C/s, then decreased at 1,5 C/s to 40°C. The fluorescence was measured continuously. The melting peaks were generated using LightCycler software by plotting the first negative derivative of the fluorescence over the temperature versus the temperature (-dF/dT).

Ability of the designed set of primers to detect and discriminate AMPV subtypes A and B. The ability to detect and differentiate the AMPV subtypes of the SYBR Green I RRT-PCRs was investigated using RNA extracted from the AMPV strains above mentioned.

Assays efficiency. RNA extracted from the AMPV of the B strain IT/Ty/Vr240/87 has been tenfold serially diluted. Each dilution was analyzed in replicate by RRT-PCR. By plotting the log dilution against the corresponding threshold cycles (Ct) value, a standard curve for each set of primers was obtained. Subsequently efficiency and error values were calculated using the slopes of each standard curve with the aid of the LightCycler 480 version 1.5 software (Roche).

Molecular Beacon one-step RRT-PCR

A further RRT-PCR protocol was developed using a probe-based detection system with the set of primers based on the SH gene sequence previously tested in the SYBR Green I RRT-PCR. Two subtype-specific molecular beacon probes were designed using Beacon Designer 7.1 software. The probes AMPV subtype A and B were respectively labeled with HEX and FAM dyes, which can be differentiated by their maximum emission wavelength, allowing their use in a duplex assay.

Molecular Beacon one-step RRT-PCR was performed using a LightCycler 480 (Roche, Basel, Switzerland). The final reaction mixture volume of 10 µL contained 0.25 µL of SuperscriptIIIRT/Platinum Taq mix (Superscript® III Platinum® One-Step Quantitative RT-PCR System, Invitrogen, Carlsbad, CA, USA), 5 µL of 2X Reaction Mix, 0,5 µL of each primers (10 µM) (forward and reverse), 0, 38 µL of each probe (20 µM), 2,24 µL of RNasi-free water and one µL of template RNA. All samples were run in duplicate. Thermocycling parameters were as follows: 45°C, 30 min (reverse transcription); 95°C, two min (RT inactivation and activation of Taq DNA polymerase); 45 cycles combining 95°C, 15 s (denaturation), 45°C 30 s (annealing and fluorescence data collection) and 72°C 0,5 s (extension step). Then the temperature was decreased at 1,5 C/s to 40°C. Data were analyzed with the LightCycler 480 version 1.5 software (Roche). For each detector (HEX and FAM) the baseline and threshold values were determined

automatically and in the case of duplicate samples a mean value was considered as the result.

Assay specificity. The specificity of the assay was evaluated using 16 AMPV strains belonging to subtypes A and, in the majority, B, one AMPV of C subtype, and three non-AMPV avian respiratory viruses, avian paramyxovirus 1, avian coronavirus, and infectious laryngotracheitis virus.

Assay sensitivity. The viral RNA was extracted from the AMPV B strain IT/Ty/Vr240/87 and from the AMPV A strain IT/Ty/A/259-01/03 and then serially diluted. Each dilution was run in duplicate. A standard curve for each subtype was generated with the resulting Ct values, in order to determine the efficiency, accuracy, and sensitivity of the assay by using the LightCycler 480 version 1.5 software (Roche).

Assay detection limit and PCR efficiency. The detection limit of the assay was evaluated using a circular plasmid DNA containing part of the SH gene of the AMPV strain of subtype B IT/Ty/Vr240/87. The plasmid DNA was constructed by inserting part of the SH gene into pCR® 2.1 TOPO vector from TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of the constructed plasmid DNA was determined by a spectrophotometer and the DNA copy number was calculated following the formula described by Ke *et al.* (8). After quantification, DNA plasmids were diluted serially in ten-fold dilutions, ranging from 2, $52X10^6$ to 2.52 copies of DNA plasmid/reaction. Each dilution was subjected to Molecular Beacon one-step RRT-PCR using Kapa Probe Fast qPCR Master Mix according to the manufacturer's instructions. Thermocycling parameters were as follows: 95°C, three min; 45 cycles combining 95°C, three s (denaturation), 50°C 30 s (annealing and fluorescence data collection) and 72°C five s (extension step). Then the temperature was decreased at 1,5 C/s to 40°C. Data were analyzed with the LightCycler 480 version 1.5 software (Roche). A standard curve was generated using the resulting Ct values and efficiency and error value were calculated using the LightCycler 480 version 1.5 software (Roche).

Comparison between the Molecular Beacon one-step RRT-PCR and an established RT-nested PCR

The RNA extracted both from virus strains or oro-pharyngeal swabs, sampled during two experimental infections, were analyzed in parallel by Molecular Beacon one-step RRT-PCR and RT-nested PCR (10). RNA was extracted from the AMPV strains IT/Ty/Vr240/87 (subtype B) and IT/Ty/A/259-01/03 (subtype A), and later tenfold serially diluted. Samples from infected birds were obtained during two different experimental infections of poultry with AMPV performed in isolation units. In the first trial ten one-day-old SPF chickens were inoculated with an AMPV subtype A, strain LTZ, and rhino-pharyngeal swabs were collected at four, five, six, seven, and eight days post infection (p.i.). In the second trial eight commercial turkeys were inoculated at 19 days of age with the subtype B strain IT/Ty/Vr240/87. Sampling was carried out on days three, five, seven, and ten p.i. RNA extraction from swabs and RT-nested PCR were performed according to Cavanagh *et al.* (2).

RESULTS

SYBR Green I one-step RRT-PCR

Ability of the five sets of primers to detect and discriminate AMPV subtype A and B. All sets of primers were able to detect the strains analyzed, producing Ct ranging from 17.65 to 35. In the case of AMPV subtype A one of the pairs of primers N-based yielded the lowest Ct value (23.37) whereas the lowest Ct value for subtype B was generated by the set of primers SH-based (17.65 and 18.72). The set of primers based on the F gene gave the highest Ct-values both for subtype A (35) or B (23.08 and 25.45). The melting curve analysis revealed that all RRT-PCR products have generated an unambiguous melting temperature (Tm) except those amplified by the G-based primers set, which presented two Tm for AMPV subtype A. In order to be able to differentiate melting peaks generated by subtype A, from those produced by subtype B, 1 C° of difference between the Tm was looked for. The only set of primers unable to give a significant difference between Tm of subtype A and B was one set designed in this study and based on the N gene.

Assay efficiency. The lowest error value was obtained with the SH-based primer set and the best efficiency (closest to two) with one of the set of primers N-based. Nevertheless the primers in SH presented a standard curve with a better linearity thanks to the really low error value (0,00217). Furthermore the SH primers set yielded the lowest Ct values. The primers designed in the F gene have given both the worst efficiency and error values. Specific Tm were obtained only from dilutions lower than 10^{-3}.

Molecular Beacon one-step RRT-PCR

Assay specificity. The AMPV strains of subtype A and B tested were amplified and detected with a specific probe, generating Ct ranging from 14.39 to 24.13. None heterologous signal was read by the FAM and HEX specific detectors. The amplification did not occurre when AMPV of subtype C or non-AMPV avian respiratory viruses were tested.

Assay sensitivity. The RRT-PCR was able to detect signals up to 10^{-4} and 10^{-6} dilutions, when AMPV of subtype A or B were respectively used as

template. The standard curves have revealed efficiencies of 113% or 73% for AMPV of subtype A or B respectively. The error values were within the optimal ranges (0,02 and 0,04 respectively).

Assay detection limit and PCR efficiency. The detection limit of the assay was 25.2 copies of plasmid DNA. The amplification's efficiency was close to two (1,982) and the error value (0,0223) was optimal, making the standard curve appropriate for absolute quantification. Yet the real detection limit is unknown, because concentrations lower than 25.2 copies/µL weren't tested.

Comparison between the Molecular Beacon one-step RRT-PCR and an established RT-nested PCR. Molecular Beacon one-step RRT-PCR, when compared to RT nested-PCR, has exhibit a sensitivity 100 to 1000 or 1000 to 10000 times higher for detection of AMPV of subtypes B or A, respectively. The analysis of RNA samples extracted from rhino-pharyngeal swabs, confirmed the greater sensitivity of the RRT-PCR assay. Out of 50 samples collected from birds experimentally inoculated with AMPV of subtype A, only one was positive to the RT-nested PCR, while 45 to the RRT-PCR. Analogously, five samples out of 32 collected from birds inoculated with AMPV of subtype B, were positive to RT-nested PCR, whereas 31 out 32 to the RRT-PCR.

DISCUSSION

Thanks to low cost and to the ease of development, the first choice system of detection used in the design of a new Real-time PCR assay, in our study, was SYBR Green I. The use of this chemistry helped to verify the proper design of the primers and find the best set of primers (SH-based primers), but the lack of unambiguous Tm at low RNA concentrations has hindered its use as quantification assay. Hence in later experiments SYBR Green I was replaced with two subtype-specific molecular beacons.

The detection limit of the molecular-beacons assay was assessed by analyzing a DNA plasmid containing part of the SH gene of AMPV of subtype B, and resulted in detection of 25.2 copies of DNA plasmid. This value makes this test about four times more sensitive than the Real-time PCR developed by Guionie *et al.* (7) and 24 times more sensitive than the RT-PCR designed by Cecchinato *et al.* (3). Moreover the standard curve obtained with the Ct values, could be useful for absolute quantification of viral RNA in field and experimental samples.

Compared to an established protocol of a subtype A and B specific RT-nested PCR (10), the real-time PCR developed herein, showed a greater sensitivity. Different results were obtained from Ferreira *et al.* (5) that found their RRT-PCR protocol to have sensitivity similar to that of conventional RT-PCR.

Despite the efficacy of molecular-beacons, which depends mostly on genetic stability of the sequences, our assay was able to detect all tested Italian AMPV strains of subtype A and B, including AMPV of subtype B strains belonging to two different genetic clusters (4), without detecting non-AMPV avian respiratory viruses. These characteristics make the molecular-beacon RRT-PCR implemented in this study a powerful diagnostic tool suitable to be used in Italy. The next step will be to analyze strains of AMPV subtype A and B originating from different parts of the world in order to verify the possibility to apply this assay in other geographical areas.

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NEW APPROACHES IN THE PREVENTION OF VELOGENIC NEWCASTLE DISEASE IN MEXICO

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OBJECTIVE

The objective of this field study was to assess, through challenge studies, the level of protection elicited by a vector HVT Newcastle vaccine when used at the hatchery in association with a live enterotropic Newcastle disease virus (NDV) vaccine as a basic NDV vaccination program in commercial broiler flocks raised in areas of high NDV field pressure in comparison with conventional live and inactivated NDV vaccination programs.

MATERIALS & METHODS

Broilers. A total of 240 broilers of different ages (From 17 to 35 d of age) from eight commercial flocks were taken from farms located in several geographic regions in Mexico, representing over seven million birds raised in areas of high NDV field challenge. Random selections of groups of ten broilers vaccinated with the experimental vaccination program were taken to a laboratory for a challenge study. Ten groups of broilers randomly selected from different poultry houses vaccinated with standard NDV vaccination programs were used as controls.

Vaccines and vaccination programs. The experimental vaccination program was composed of a vector HVT-NDV vaccine (Vectormune® HVT-NDV) injected subcutaneously together with a live NDV vaccine administered via spray at one d of age (strain PHY-LMV 42). Conventional NDV vaccination programs with live and inactivated NDV vaccines were used as controls. The field revaccination with conventional live or inactivated NDV vaccines was done using vaccines from different commercial sources and administered at different ages (Table 1).

Challenge. A total of twelve challenge studies were conducted in this study. Each group of ten birds was allocated in an isolating unit type Horsfall-Bauer in addition with five SPF birds of the same age as positive controls. Each bird from each group, including the contact SPF birds, received 0.3 mL of the Chimalhuacan NDV velogenic strain, with a titer of 10^6 EID_{50} (Class II, Genotype V, with an ICPI of 1.89) via ocular route.

Parameters. The birds were observed for 14 d post-challenge (PC) for clinical signs and mortality. Blood samples were taken the day of the challenge for serological testing (Idexx ELISA and HI using four HU in 1/10 serial dilutions). Viral excretion was also evaluated at six d PC using two pools (each of five swabs) of tracheal and cloacal swabs taken for RT-PCR analysis.

RESULTS

Livability. None of the broilers in the experimental or the control groups had clinical signs or died. All susceptible contact SPF birds died within three to six d PC.

Serology. In the experimental vaccination groups, the serological response as detected by the HI and ELISA was low and in some cases negative at pre-challenge sampling and within the standard serological values after challenge, but then lower than the values observed in the conventional vaccination groups.

Virus excretion. A significantly lower reduction of excretion of the challenge virus was observed in the experimental vaccination groups in comparison with the conventional NDV vaccination groups.

DISCUSSION

There was 100% protection in all challenged groups without clinical signs or mortality either within the experimental treatment groups vaccinated with the

vector HVT-NDV vaccine or the control treatment groups with the conventional NDV vaccination programs. The SPF contact controls had 100% clinical signs and lesions of ND and died within three to six d post-challenge.

The group of birds that received the vector HVT-NDV vaccine in addition to live NDV vaccines did generate a low serological response either in the HI test or the ELISA (Idexx). In these studies, the serological response was not related with protection as it has been shown in other studies where live and inactivated NDV vaccines have been used. This issue is relevant for the interpretation of the serological results when the vector HVT-NDV vaccine is used in commercial poultry operations.

Another important observation was that groups vaccinated with the vector HVT-NDV vaccine excreted a significantly lower amount of NDV used for challenge than the control groups vaccinated with conventional NDV vaccination programs. This experimental vaccination program has a strong potential to significantly reduce the transmission of velogenic NDV in a more efficient way than the conventional NDV vaccination programs with inactivated vaccines, offering a NDV control approach more efficacious than the ones currently used.

We conclude that the vector HVT-NDV vaccine is safe and more effective as part of an NDV vaccination program in commercial broilers raised in areas with high field NDV challenge.

Table 1. Newcastle disease vaccination programs in experimental and control groups.

Trial	NDV Vaccination Program Experimental	NDV Vaccination Program Control
1	rHVT-NDV, SQ, 1-day-old Live virus, Spray, 1 & 12 days of age	NDV Inactivated, SQ, 1-day-old Live virus, Spray, 1 & 12 days of age
2	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/Spray at 1, 12 & 23 days of age	NDV Inactivated, SQ, 1 & 12 days of age Live virus, Spray/ED/Spray at 1, 12 & 23 days of age
3	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/DW at 1, 12 & 28 days of age	NDV Inactivated, SQ, 1 & 12 days of age Live virus, Spray/ED/DW at 1, 12 & 28 days of age
4	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 15, 24 & 35 days	rHVT-NDV+ SB-1, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days
5	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 15, 24 & 35 days	rHVT-NDV+ SB-1, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days
6	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 15, 24 & 35 days	rHVT-NDV+ SB-1, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days
7	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days	NDV Inactivated, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days
8	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days	NDV Inactivated, SQ, 1 & 12 days of age Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days
9	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days	NDV Inactivated, SQ, 1 & 12 days of age Live virus, Spray/ED/Spray/Spray at 1, 12, 24 & 35 days
10	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED at 1 & 8 days of age	NDV Inactivated, SQ, 8 days of age Live virus, Spray/ED/DW at 1, 8 & 18 days of age
11	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED at 1 & 8 days of age	NDV Inactivated, SQ, 1 & 8 days of age Live virus, Spray/ED at 1 & 8 days of age
12	rHVT-NDV, SQ, 1-day-old Live virus, Spray/ED at 1 & 8 days of age	NDV Inactivated, SQ, 1 & 8 days of age Live virus, Spray/ED at 1 & 8 days of age

ED = Eye Drop route of live NDV vaccination. DW = Drinking water route of live NDV vaccination.

VACCINE INTERACTION AND PROTECTION AGAINST VIRULENT AVIAN METAPNEUMOVIRUS (AMPV) CHALLENGE AFTER COMBINED ADMINISTRATION OF NEWCASTLE DISEASE AND AMPV LIVE VACCINES TO ONE-DAY OLD TURKEYS

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SUMMARY

The combined administration of Newcastle disease (ND) and avian metapneumovirus (AMPV) live vaccines in turkey hatcheries is advantageous, but compatibility has not yet been experimentally demonstrated. To investigate any possible interference between the two vaccines, AMPV subtype B live vaccine strain VCO3 was given to one d old turkeys either alone or in combination with two different ND vaccines of the B1 and VG/GA strains. AMPV and NDV post-vaccination shedding and humoral immune response were assessed. Birds were protected from virulent AMPV challenge and differences in clinical signs between groups vaccinated with AMPV vaccine alone or in combination with ND vaccines were not statistically significant. Furthermore there was not interference in vaccines replication in the respiratory tract. Further studies are required to understand if protection after NDV challenge is affected by NDV and AMPV combined vaccination.

INTRODUCTION

The turkey industry involves a very large number of animals raised in highly densely populated poultry areas; these conditions facilitates the diffusion of infectious diseases, making vaccination programs and biosecurity essential to reduce the risk of disease outbreaks. Vaccination against ND is compulsory in Italy (7), and turkeys are also frequently vaccinated against turkey rhinotracheitis (TRT) (2).

ND, caused by avian paramyxovirus-1 pathogenic strains, is one of most feared diseases in poultry. Highly pathogenic strains cause serious economic losses due to high mortality and morbidity. Application of eradication programs and trade restrictions apply to the country where the outbreak has occurred. AMPV causes turkey rhinotracheitis, one of the most important viral infections in turkeys, and it is widespread practically almost all over the world. The severity of the disease and the mortality rate are largely influenced by secondary bacterial infection. Both diseases are kept under control in turkey farms by using live vaccines administered during the first days of life with at least an interval of seven d between the vaccinations so as to avoid possible interference between the vaccines. A vaccination program including a simultaneous administration of both vaccines in the hatchery would have several practical, economic, and biosecurity advantages. Such a modification requires a study about vaccine compatibility.

The aim of the present study is to evaluate, under experimental conditions, the interaction between two different NDV vaccinal strains and one AMPV vaccine strain, co-administered in one d old turkeys.

MATERIALS AND METHODS

The trial was performed in biological isolation systems and had the total duration of 32 d. Eighty one d old commercial turkeys were divided into six groups and vaccinated as follow:

- TRT group (16 birds) – vaccinated with AMPV subtype B strain VCO3
- B1 group (8 birds) – vaccinated with NDV strain B1
- VG/GA group (8 birds) – vaccinated with NDV strain VG/GA

● TRT+B1 group (16 birds) – vaccinated with AMPV subtype B strain VCO3 and NDV strain B1, co-administered
● TRT+VG/GA group (16 birds) – vaccinated with AMPV subtype B strain VCO3 and NDV strain VG/GA, co-administered
● CONTROL group (16 birds) – treated with sterile water

The vaccines were prepared as recommended by the manufacturer and given according to the commercial dose. Turkeys were monitored daily for post-vaccination (p.v.) clinical signs.

Oro-pharyngeal swabs were collected at 2, 4, 6, 8, 10, 14, 18, 22, 26, and 30 d p.v. to investigate the colonization and replication of vaccine viruses in target tissues by reverse transcriptase real time polymerase-chain reaction (RT-Real Time PCR). For this purpose, RNA was extracted using the method described by Li *et al*. (5) and for NDV Real Time RT-PCR (RRT-PCR) was performed using the method described by Cattoli *et al*. (1). For AMPV, a protocol of RRT-PCR involving the use of a subtype B Molecular Beacon probe was used, designed on the sequence of the gene coding for small hydrophobic protein (3).

Blood samples were obtained at 7, 14, 21, and 28 d p.v. for detection of antibodies anti-AMPV by ELISA (ELISA kit Flockchek® APV Ab, IDEXX Laboratories), and anti-NDV by hemagglutination inhibition (HI) (8). The mean titers were compared using the Student t test and the difference was considered statistically significant with P value < 0.05. At d 21 p.v., eight animals from groups TRT, TRT+B1, TRT+VG/GA, and CONTROL were moved to other isolation units and challenged with virulent AMPV subtype B strain IT/Ty/Vr240/87. Each bird received 3.77 $\log_{10}$ CD_{50} by eye drop. From d one post-infection (p.i.) until the end of the trial, birds were monitored daily for clinical signs assigning a score to each bird, following the method described by Naylor and Jones (6). The comparison between groups was performed using the Mann Whitney U test. The difference was considered statistically significant with P-value <0.05.

RESULTS and DISCUSSION

No clinical signs were observed after vaccination applied either in single (AMPV or NDV) or in combination (AMPV+NDV).

Numbers of positive birds/day of sampling to the Real Time RT-PCR for NDV were comparable in all vaccinated groups. All the birds shed at least once four d p.v. The number of positive birds/group decreases significantly after the tenth d p.v. The total number of positive birds to RRT-PCR for AMPV differs only slightly among the three vaccinated groups (Table 1). All birds shed at least once at the fourteenth day p.v.

No statistically significant difference ($p > 0.05$) between antibody titers of the AMPV vaccinated group and the CONTROL group was found until d 21 p.v. At 28 d p.v. the mean antibody titers appears to be statistically higher in groups vaccinated in combination when compared to the CONTROL group and the TRT group ($p < 0.05$). These results are similar to those observed in chickens by Ganapathy *et al*. (4), and need further investigation.

The mean NDV-HI titers did not show significant differences between groups until d 21 ($p > 0.05$). At d 28 the control group and the vaccinated groups differed significantly ($p < 0.05$). Statistically significant differences were not observed between groups that received single or in combination vaccines for both vaccine strains.

After challenge with field AMPV strain, unvaccinated and challenged birds showed clinical signs with a mean score of 15.25, significantly higher than all vaccinated groups. Statistically significant differences in clinical scores were not found between the groups vaccinated in single or in combination (Figure 1).

CONCLUSIONS

In conclusion, it appears that concurrent vaccination of one d old commercial turkeys with AMPV VCO3 vaccine strain and NDV B1 or VG/GA vaccine strains gives the same protection after AMPV challenge. Furthermore, the AMPV vaccine does not interfere with ND vaccines replication in the respiratory tract.

Further studies are required to understand if protection after NDV challenge is affected by NDV and AMPV combined vaccination. As a matter of fact, the same shedding values of vaccine NDV in the different groups let us suppose that colonization and replication in the respiratory tract, and consequently the stimulated immunity, has not been reduced by the simultaneous administration. This is likely to be true either for NDV B1strain, that exhibit mainly respiratory tropism, or for NDV VG/GA strain that shows mainly enteric tropism**.**

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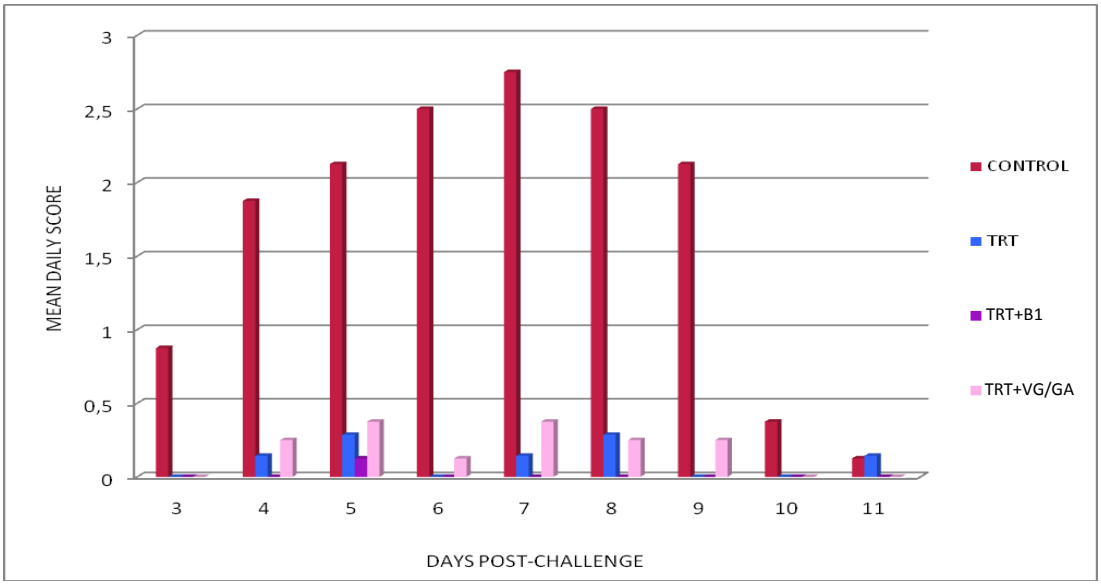
Table 1. Number of positive birds/day to the Real time RT-PCR for AMPV vaccine virus.

Days post-vaccination	TRT	TRT+B1	TRT+VG/GA	CONTROL
2	0*	1	0	0
4	1	0	0	0
6	4	1	3	0
8	2	0	4	0
10	5	2	5	0
14	7	7	6	0
18	4	8	4	0
22	0	4	1	0
26	2	1	0	0
30	2	2	0	0
TOTAL	**27**	**26**	**23**	**0**

***Number of swabs out of 8 from which AMPV was detected

Figure 1. Mean daily clinical scores observed in groups challenged with AMPV subtype B.



THE USE OF ELISA (BIOCHEK) TO DETECT ANTIBODIES FOLLOWING VACCINATION WITH DIFFERENT RECOMBINANT HVT VECTORED VACCINES FOR NDV, ILTV, AND IBDV

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INTRODUCTION

The ELISA test is one of the most widely used diagnostic technologies available. In addition to diagnostics, it has been proven as a practical means to evaluate the success of vaccination. In determining the success of an individual program, it is crucial to know what results are expected following a particular vaccination. For many of the conventional live and inactivated vaccines, ELISA (BioChek) guidelines have been reported (3). However, there is now a new generation of vaccines. These vaccines are recombinant viral vector vaccines, containing as vectors fowl pox virus (FPV) or herpesvirus of turkey (HVT). Recombinant vaccines based on HVT as the vector expressing genes that code for specific ILTV, NDV, or IBDV antigens, for example, to induce immunity for ILTV, NDV, or IBDV are more recent vaccines. With the use of these new recombinant vectored vaccines, new practical guidelines are needed to assist with interpretation of ELISA results. Results have been previously reported for the recombinant (r) HVT-ILT, Innovax-ILT® using the BioChek ILT ELISA and for the rHVT-IBD vaccine, Vaxxitek® HVT+IBD, using two different IBD ELISA kits (1, 2). This paper highlights the results observed on BioChek ELISAs after vaccination with the recombinant HVT vectored vaccines and provides guidelines to assess successful vaccination and aid in the interpretation of field results.

PROCEDURES

Broilers were vaccinated either *in-ovo* at 18 d of embryogenesis or subcutaneously at one d of age with different recombinant HVT vectored vaccines containing immunogenic proteins for NDV, IBD, or ILT. At different intervals post-vaccination, sera was collected and assayed for NDV, IBD, or ILT antibodies using ELISA and virus neutralization (VN) or hemagglutination inhibition (HI) assays.

RESULTS AND CONCLUSIONS

The BioChek IBD, NDV, and ILT ELISA kits are suitable for monitoring vaccination with the respective HVT vectored recombinants. There was a good correlation between the conventional serology (VN or HI) and ELISA (data will be presented). Furthermore, the ELISAs were capable of differentiating between normal vaccination response and field challenge. The ideal time for monitoring is after 35 d. Mean titers after vaccination with the recombinant HVT vector vaccines tend to be very low, particularly the rHVT-ND and rHVT-ILT vaccines (Table 1). It has been reported that for rHVT-ILT, the percent positive is more useful in accessing vaccination and/or challenge (2). For rHVT-ND, the trend was similar.

The guidelines provided below are based on a limited number of studies with currently available and licensed vaccines. More data is still needed to validate these trends and confirm the results. In addition, new vaccines are continually being developed which may show different ELISA results post-vaccination and post-vaccination/challenge. As vaccines are developed, it is important to evaluate and develop guidelines to assist with interpretation. In conclusion, these results show that BioChek ELISAs are capable of detecting and differentiating the current rHVT - (ILT, IBD, and ND) vaccines from field challenge.

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Table 1. BioChek ELISA guidelines for interpretation in broilers vaccinated with recombinant HVT vectored vaccines.

	IBD	**ILT**	**NDV**
Vaccine	**rHVT-IBD**	**rHVT-ILT**	**rHVT-ND**
Ideal time to test	35-55 d	>40 d	> 40 d
Mean Titer	500-3000	500-1500	500 - 1500
% Positive	80-100	10-60	10-60
%CV	> 45	--	--
VI	10-50	< 40	< 40
	Suspect challenge		
Mean Titer	> 4000	> 2000	> 3000
% Positive	100	$\geq$ 80	$\geq$ 80
%CV	< 45	--	--
VI	$\geq$ 100	$\geq$ 50	$\geq$ 50

CURRENT ALTERNATIVES TO ANTIBIOTICS

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SUMMARY

Antibiotic use in poultry has been around since the 1950s. It was discovered that antibiotics added in poultry diets would improve poultry growth and feed efficiency. In fact, some would suggest that the success of the poultry industry since the 1950s is due to two things: vaccination and antibiotics. Not long after the start of use of antibiotics in poultry, the idea of developing resistance to antibiotics became a concern. There has been much discussion on rather using antibiotics in poultry feed will lead to resistance in human medicine. This has led to a whole new segment of the poultry industry: antibiotic free birds and organic birds. Customers will often pay a higher price to have antibiotic free poultry because of the concerns with antibiotic resistance.

With the development of the antibiotic free segment of the poultry industry and the concern with antibiotic resistance as well as the constant threat of banning antibiotics in poultry feed, there has been a need for alternatives to antibiotics. Over the years, many alternatives to antibiotics have been developed and explored. Some of these alternatives are probiotics, prebiotics, organic acids, enzymes, and essential oils. Probiotics have been around for a long time; the definition usually used for probiotics is "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (3). Prebiotics are non-digestible food ingredients that stimulate the growth and/or activity of bacteria in the digestive system in ways claimed to be beneficial to health. They were first identified and named by Marcel Roberfroid in 1995 (4). There are many commercially available prebiotics and probiotics. The companies selling these products have generated a good deal of research to support their products.

Organic acids have specific antimicrobial activity and are pH dependent. Organic acids have been shown to have an effect on acid-intolerant species like *E. coli*,

Salmonella, and *Campylobacter*. Other beneficial claims of organic acid include reduction in digesta pH, increased pancreatic secretion, and trophic effects on gastrointestinal mucosa.

Enzymes usually don't have a direct effect on gut microbial numbers, but does have an effect on improving nutrient digestibility. Enzymes increase the level of small and medium size chain carbohydrates that beneficial microflora prefer to use. Enzymes also work by reducing digesta viscosity, which increases the flow through the upper gut, which will reduce the possibility of proliferation of bacteria in that part of the gut (5).

Essential oils are a mixture of fragrant, volatile compounds, named after the aromatic characteristics of plant materials from which they can be isolated. Essential oils have long been recognized for their anti-microbial activity (2). Essential oils enhance production of digestive secretions, stimulate blood circulation, exert antioxidant properties, reduce levels of pathogenic bacteria, and may enhance immune status (1).

There has been a great deal of research on many products within each of these categories. These products work on producing a healthy gut. A healthy gut is usually measured by the status of the gut microbotia and gut flora. The gut is the biggest immune organ of the body, and since many of these products affect gut integrity, they also have an effect on the immune system. However, the research results have been inconsistent, leading to some confusion on what benefits these products can have in poultry diets and if some are good alternatives to antibiotics. This paper will explore some of these results and what the future may hold for using these products as alternatives to antibiotics.

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SULFONAMIDES AS PROPHYLACTIC AND THERAPEUTIC AGENTS FOR POULTRY

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INTRODUCTION

The sulfonamides hold a special place as prophylactic and therapeutic agents in the history of human and veterinary medicine. In 1932 a German physician and researcher named Gerhard Domagk discovered that a red dye known as prontosil rubrum protected laboratory animals from infections with Gram positive cocci. It was later demonstrated that prontosil was also effective in the treatment of human bacterial infections (10). Subsequently, French researchers Jacques and Therese Trefouel (husband and wife) discovered that the antibacterial activity of prontosil against human infections was due to an intermediate metabolite produced by the body and determined to be sulfanilamide (p-amino benzene sulfonamide). This was confirmed in 1936 by Dr. Albert Fuller who detected sulfanilamide in the urine of patients treated with prontosil (10). This discovery paved the way for the development and introduction of a large number of sulfonamides with varying pharmacodynamic and pharmacokinetic properties for use in human and veterinary medicine, all of these sulfonamides are derivatives of the original sulfanilamide.

MODE OF ACTION

The sulfonamides block the synthesis of folic acid by bacteria, which unlike eukaryotic cells, they must synthesize it in order to produce the purines required for the formation of nucleic acids. More specifically, due to their chemical structure similarity to p-amino benzoic acid (PABA), sulfonamides block the production of dihydroteroic acid from pteridine plus PABA catalyzed by dihydropteroic acid

synthetase, this competitive blocking of PABA can be reversed by an excess production of PABA at the infection site (one reason for removing tissue exudates and necrotic debris when animals are treated with sulfonamides).

POTENTIATION BY DIAMINOPIRYMIDINES (DAPs)

After resistance to sulfonamides became a problem, their combined used with diaminopirymidines (DAPs) such as trimethoprim and ormethoprim as potentiating agents became well established. DAPs further block the production of folic acid by bacteria by preventing the formation of tetrahydrofolic acid from dihydrofolic acid catalyzed by dihydrofolic acid reductase (DFAR). Selective antibacterial activity is due to the greater affinity of DAPs for bacterial DFAR vs. mammalian or avian DFAR (17).

Sulfonamides are bacteriostatic when used alone but in many cases their activity becomes bactericidal when combined with DAPs. The enhanced activity of the potentiated sulfonamides against fowl cholera of turkeys, and *E. coli*-induced airsacculitis and coccidiosis of chickens has been documented in the scientific literature (19, 12, 14, 15).

SPECTRUM OF ACTIVITY

The sulfonamides have a broad spectrum of activity that includes Gram positive and Gram negative bacteria, fungi like *Pneumocystis* spp., as well as protozoan parasites such as *Toxoplasma* spp., *Eimeria* spp., and *Cryptosporidium* spp. Bacteria that generally exhibit good sensitivity include *Bacillus* spp., *Brucella* spp., *Erysipelothrix rushiopathiae*, *Listeria monocytogenes*, *Nocardia* spp., pyogenic strains of *Streptococcus* spp., and *Chlamydia* spp. (17).

Since sulfonamides have been used for over 50 years, a significant level of resistance has developed in certain genera of bacteria while others like the *Mycobacterium* spp., *Mycoplasma* spp., *Rickettsia* spp., *Pseudomonas aeruginosa*, and spirochetes are completely resistant. In order to enhance their efficacy against resistant strains of microorganisms, sulfonamides have been combined with DAPs and these combinations are the most commonly used at the present time.

PHARMACOLOGICAL STUDIES

Published studies evaluating pharmacodynamic and pharmacokinetic parameters of sulfonamide administration in poultry are scarce. In an old study, the blood concentrations of active principle for seven sulfonamides included in the feed at a fixed rate (0.2%) were measured in chickens and turkey poults (9). The researchers found that of the seven sulfonamides evaluated, only three (sulfamethazine, sulfamerazine and sulfadiazine) maintained therapeutic blood concentrations throughout the test period.

The pharmacokinetics of each sulfonamide are different and must be considered before implementing a therapeutic regimen. For example, in studies with pullorum disease it was discovered that sulfamerazine produced better results when administered in the feed vs. the drinking water (18). In contrast, sulfaquinoxaline (SQ) was found to be more effective for reducing mortality from pullorum disease when administered in the drinking water vs. the feed (6).

In a more recent study (1), the pharmacokinetic properties of a newly approved European potentiated combination of sulfadiazine and ormethoprim were evaluated in broiler chickens, the new combination was administered both orally and parenterally (IV injection) and the plasma concentrations of both drugs were determined by HPLC and the pharmacokinetic parameters calculated. The researchers found that regardless of the route of administration used, both drugs were quickly cleared from the plasma. The mean half-life for trimethoprim and sulfadiazine was 1.61 and 3.2 hours, respectively. The apparent volumes of distribution (2.2 and 0.43 L/kg, respectively) indicated that the tissue distribution of trimethoprim was more extensive than that of sulfadiazine. The estimated oral bioavailability was approximately 80% for both drugs.

USE OF SULFONAMIDES IN POULTRY

Prophylactic use. As early as 1939, Dr. P.P. Levine, a poultry veterinarian and parasitologist at Cornell University, studied and reported on the effect of sulfanilamide on coccidiosis of poultry (11). The first prophylactic use of a sulfonamide in poultry involved the inclusion of sulfaquinoxaline (SQ) in broiler chicken feed for the prevention of coccidiosis (5). The importance of this landmark discovery has been recently emphasized by H.D. Chapman in a manuscript (2).

Therapeutic use. Some of the first reports on the therapeutic efficacy of the sulfonamides against poultry diseases dealt with the treatment of coccidiosis (11, 7, 5) fowl cholera (8), and pullorum disease (18, 6). Years later the effectiveness of sulfonamide therapy for the control of infectious coryza in chickens became well documented (16). To this date those remain the diseases against which sulfonamides are most frequently used although sulfonamides have been used successfully to treat other types of bacterial diseases, like those caused by atypical *Pasteurella* infections in chickens (3) and *Pasteurella anatipestifer* in ducklings (4).

When sulfonamides are used as therapeutic agents some factors must be considered to maximize their therapeutic efficacy and minimize the risk of adverse effects. For example, the use of SQ in the feed at an inclusion rate of 0.5% has produced hypoprothrombinemia and delayed clotting after beak trimming in ducklings. This adverse effect can be prevented by the addition of vitamin K to the diet (4).

For the treatment of coccidiosis in chickens, the type of sulfonamide chosen and the species of *Eimeria* causing disease may be important considerations, for example, Mathis and McDougald found that SQ and SQ plus pyrimethamine were effective to control infections induced by *E. acervulina* but not as effective to control infections induced by *E. tenella* (13). Earlier and more extensive studies showed that sulfadimethoxine at 0.05% in the drinking water was highly effective against all the most common *Eimeria* spp (either singly or mixed) that infect broiler chickens when administered three days after infection and for six consecutive days (14). The same researchers demonstrated the therapeutic efficacy of sulfadimethoxine at 0.025% in the drinking water for the treatment of turkeys infected by single or mixed infections of *E. gallopavonis*, *E. meleagrimitis*, and *E. adenoides*.

PRACTICAL CONSIDERATIONS

When sulfonamide use in poultry is considered, the following points must be carefully examined in order to increase its therapeutic effectiveness.

1) An accurate disease diagnosis is the most important initial step. Diseases like coccidiosis, infectious coryza, *Pasteurella* infections, erysipelas and in certain cases, infections by sensitive species of *Salmonella* and strains of *E. coli*, tend to respond well to sulfonamides.

2) After many decades of sulfonamide use, resistance has become a significant problem, thus, bacterial isolation and sensitivity are recommended. Treatment can be initiated right away if samples are collected at that time for bacterial culture and sensitivity testing.

3) The use of potentiated sulfonamides can increase their therapeutic efficacy as the spectrum of activity is enhanced and the resistance problems reduced.

4) When drinking water administration is considered, the pH of the water is an important consideration as sulfonamides are known to be more efficacious when administered in alkaline vs. acid water. The propensity of certain sulfonamides administered via the drinking water to precipitate in the tubules of the kidneys may be reduced by combining two or more of them and benefiting from the law of independent solubility which states that the solubility of each sulfonamide in a mix is independent of each other.

5) As previously discussed, the pharmacokinetics of each sulfonamide are different and must be considered before implementing a therapeutic regimen.

6) As sulfonamides are frequently administered in an intermittent schedule, (i.e., three days on/two days off; two days on/two days off, etc.) it is very important that the birds have free access to drinking water. This will aid in minimizing any potential adverse effects.

7) Residues can be a problem when sulfonamides are used as therapeutic agents in poultry. It is important that withdrawal times are strictly followed and that all feed or drinking water that contained sulfonamides is properly flushed out or removed from the system.

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ANTIMICROBIAL EFFICACY OF ACIDS IN THE FEED OR DRINKING WATER OF BROILER CHICKENS

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SUMMARY

Recently, there has been a trend to move to an all natural chicken product. In doing so, we must consider natural additives which are focused on improving gut health by manipulating intestinal bacterial populations. One of the major gut health issues impacted by intestinal bacteria is necrotic enteritis. Necrotic enteritis is caused by the proliferation of *Clostridium perfringens* with subsequent production of intestinal necrosis toxins. Studies have shown that antibiotic growth promoters, direct fed microbials, prebiotics (MOS), essential oils, saponin or sapogenines (yucca and/or quillaja), acidifers, and physically activated vitamin C combined with organic acids, improve poultry health and performance while reducing or changing the intestinal bacterial profile. Acidifiers are one of the most successfully used. According to a Global Industry Analyst report published in 2010, the global market for feed acidifiers will be 1.4 billion USD by 2015. Acidifiers may be organic or inorganic compounds. The objective of this presentation is to discuss studies, which evaluated acidifiers in both feed and water application by reducing the effects of the bacterial disease necrotic enteritis.

INTRODUCTION

For poultry producers to make a profit they must produce healthy, uniform sized birds, while making sure there are no real or assumed human health concerns that are associated with limiting antibiotic resistance, and formulating diets on a least cost basis. Traditionally, antibiotics such as bacitracin methylene disalicylate, tylosin phosphate, and virginiamycin have been fed at subtherapeutic doses in the poultry industry to maintain healthy birds resulting in growth promotion and thus increase profit. The EU ban of feeding antibiotics growth promoters (AGP) to broilers, and general increased awareness among consumers has increased the demand for “antibiotic free” or “natural produced” poultry products. Thus, there is a need for alternative feed additives to AGPs. Some of these alternatives are direct fed microbials, prebiotics (MOS), essential oils, saponin or sapogenines (yucca and/or quillaja), acidifers, and physically activated vitamin C combined with organic acids. As with the AGPs, these products must work in today’s poultry production environment where feed prices are high, alternative feed ingredients are routine, marginal coccidiosis control is common (drug resistance and/or vaccination issues), heavily built-up litter is used, and the house environment is affected by high fuel prices.

Acidifiers are one of the most successfully used alternative options to AGPs. According to a Global Industry Analyst report published in 2010, the global market for feed acidifiers will be 1.4 billion USD by 2015. Acidifiers may be organic or inorganic compounds that have acidic properties. Organic acids singularly such as citric acid, propionic acid, fumaric acid, lactic acid, formic acid, and benzoic acid, or in combination have strong antimicrobial activity through disrupting the cell membrane-transport system. Organic acids in their undissociated forms pass through the cell membranes of bacteria where the acids dissociate in an alkaline medium to produce H+ ions. The H+ ions lower the pH of the cell, causing the organism to use energy to restore the normal balance. Lactic acid bacteria are able to grow at relatively low pH, and thus are more resistant to organic acids than other bacterial species. Because of this mode of action, an acidifier needs to contain a combination of organic acids that are undissociated at different pH values to allow for the antimicrobial action to be prolonged over a wider pH range.

Acidifiers have long been used in the preservation of human food and animal feed by reducing microbial and fungal levels. Studies have shown that acidifiers enhance poultry growth by improving gut health through the reduction of pH and buffering capacity of diets, improvement of pancreatic secretions that increase nutrient digestibility, or promotion of beneficial bacterial growth while inhibiting growth of pathogenic bacteria (3,4). Acidifiers can act synergistically with phytase to improve phosphorus and magnesium digestibility. Organic acid water and feed treatments have been shown to be effective in decreasing *Salmonella* colonization and horizontal transmission in broiler chickens (1,2,5). The objective of this presentation is to discuss studies examining the anticlostridial efficacy of acidifiers added to feed and water in reducing the effects of necrotic enteritis. Necrotic enteritis in poultry is caused by the proliferation of *Clostridium perfringens* with subsequent production of intestinal necrosis toxins.

MATERIALS AND METHODS

Study 1. The objective this study was to evaluate the anticlostridial efficacy of an encapsulated blend of organic and inorganic acids (citric, fumaric, malic and ortho-phosphoric) and AGP virginiamycin. Groups of ten birds were weighed and placed into cages at day of hatch. The treatments were nonmedicated, non-challenged (NMNC); nonmedicated, challenged (NMC); Acidifier 500 ppm, challenged and virginiamycin (VIR) 20 ppm, challenged. Birds were challenged at 14 d of age with *E. acervulina* and *E. maxima* and on d 19, 20, and 21 with *Clostridium perfringens*. Each treatment consisted of six replications in a complete randomized block design. The parameters measured were feed conversion, weight gain, necrotic enteritis (NE) mortality, and NE lesion scores. There were significant improvements of feed conversions and weight gains for both feed additives compared to the NMC birds. The feed conversions (d 0-28) of the NMNC were 1.461g, NMC 1.726, Acidifier 1.577g, and VIR 1.519. The average live weight gains (d 0-28) of the NMNC were 0.963 kg, NMC 0.774 kg, Acidifier 0.901 kg, and VIR 0.935 kg. Percent NE mortality was significantly less for the VIR 12 % compared to NMC 33 %. There was no significant difference in percent NE mortality between Acidifier and VIR treatments. All feed additive treatments had significantly lower NE lesion scores compared to NMC. This study demonstrated the benefits of adding this combination of acidifiers or virginiamycin into the feeds of broiler chickens exposed to *Clostridium perfringens*.

Study 2. The objective this study was to evaluate the anticlostridial efficacy of a product combining organic acids (formic, acetic, propionic, and sorbic acid) and medium-chain fatty acids (caprylic, and capric acid) (OA/MCFA) and an AGP bacitracin methylene disalicylate (BMD). This study used that same experimental design as used in Study 1. The treatments were nonmedicated, non-challenged (NMNC); nonmedicated, challenged (NMC); OA/MCFA 2 kg/t, challenged, and BMD 50 g/t, challenged. The BMD and OA/MCFA product significantly reduced the incidence of intestinal NE lesions by 22% as compared to NMC. Weight gain (d 0-28) was improved by 29%, feed conversion ratio (d 0-28) by 8%; and total NE mortality was reduced by 43%. The addition of BMD significantly improved performance and NE lesions. It is concluded that the tested OA/MCFA product shows mild anti-*Clostridium* effects *in vivo* and fits well in a total approach to control NE.

Study 3. The objective of the study was to evaluate the anticlostridial efficacy of an organic complex from the physical reaction between ascorbic, lactic, and citric acids combined with glycerin administered in the drinking water or feed, and virginiamycin. The same experimental design was used as previously mentioned The treatments were nonmedicated, non-challenged (NMNC); nonmedicated, challenged (NMC); Citrex Liquid 200 ppm or 300 ppm, Citrex Powder 400 ppm or 600 ppm and virginiamycin (VIR) 22 ppm. Using tukey (HSD) comparison of means test, the results showed that there was a significant improvement in performance (feed conversion and weight gain) for all treatments compared to the NMC treatment birds. Percent NE

mortality for NMC was 12.5 % which was significantly higher than observed with all other treatments. Percent NE mortality for all supplemented treatments was not significantly different (range 0 to 3.6%). The average NE lesion score for NMC was significantly higher than observed with all other treatments. This study demonstrated the benefits using an acidifier either in the drinking water or feed of broiler chickens exposed to *Clostridium perfringens*.

CONCLUSIONS

These three studies evaluating the anticlostridial efficacy of organic acids demonstrated the benefits of using an acidifier either in the drinking water or feed of broiler chickens exposed to *Clostridium perfringens*.

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IN VITRO STUDIES OF CELMANAX®, A YEAST PRODUCT WITH POTENTIAL FOR CONTROL OF *RIEMERELLA ANATIPESTIFER* DISEASE OF DUCKS

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SUMMARY

The reactivity of Celmanax© a hydrolyzed yeast product with *Reimerella anatipestifer* (Ra) was studied using direct agglutination and microtiter. *In vitro* reactivity resembled agglutination and flocculation depending on the serotype. Microscopic examination of yeast – *Ra* mixtures suggested a direct attachment of *Ra* to yeast with a preference for more hydrolyzed yeast cells.

Infectious serositis, a.k.a. anatipestifer infection, new duck disease, or infectious septicemia, is an important disease of commercial ducks (1). It can cause high morbidity and mortality; it has been treated with various antibiotics. Inactivated vaccines provide some serotype specific protection (2). Use of antibiotics in animals is not without controversy and the narrow spectrum of vaccine protection suggests alternative approaches to the control of this condition are warranted.

Celmanax (Cmx), hydrolyzed yeast feed additive, appears to provide protection from bacterial infections and may aid immunity a property that might be of use in controlling duck serositis. Here we used direct plate agglutination, microtiter, and microscopy to explore how this yeast reacts with *Riemerella anatipestifer*, the bacteria responsible for this disease (3).

MATERIALS AND METHODS

Live bacteria: (*Riemerella anatipestifer*, *ATCC©11845).* Bacterins: Formalin inactivated, freeze-dried *Ra* of serotypes 1, 2, 5, 6. Yeasts: Celmanax, a hydrolyzed yeast (Vi-Cor), (*Saccharomyces cerevisiae*) solid and liquid products; Product "B"; a commercially available mannan oligosaccharide; Product F (Fleischmann's Active Dry Yeast). Microtiter: Standard 96 U-bottom well plates with PBS as the diluent.

RESULTS

Log phase *R. anatipestifer* ATCC 11845 was tested for its capacity to be agglutinated by mixing it

with Cmx at 2, 20, and 40 mg/mL on glass slides. Visible agglutination was evident within a few minutes. The agglutination was dose dependent, and strongest at the high (40 mg/mL) dose. No agglutination of *Ra* was visible in the absence of Celmanax.

Studies with bacterins used working solutions containing 0.1 g solid products suspended in ten mL PBS, or 400 uL liquid Celmanax in the same volume of PBS.

In microtiter tests, *Ra* bacterins were mixed with diluted Celmanax, products "B" and "F" in order to reduce their density to allow better visualization of agglutination or flocculation. The reaction of the yeasts with bacterin was compared with the reaction of the bacterin with duck sera known to contain *Ra* antibody. The degree of reaction was estimated by visual inspection (Table 1). Based on the results it appears that *Ra* serotype 1 is reactive with each yeast but not to the same degree. Serotypes 2 and 6 were the least reactive, and there was a difference between serotype 2 Wis (Wisconsin field isolate) and 2 Ind (Indiana field isolate). The overall reactivity of various *Ra* bacterins appeared to parallel somewhat the reactivity of the immune sera although at a reduced intensity. The serotype difference by microtiter was supported by gross agglutination/aggregation results using a ceramic ring plate method (data not included).

Microscopic studies were conducted in order to support and expand what was observed using slide agglutination, microtiter, and ceramic ring plate results. The analytical strategy was to compare the processed yeasts, Celmanax and "product B" with product "F", an "active" *S. cerevisiae*, both alone an in combination with *Ra* bacterins. A standard procedure was developed by placing two uL suspensions of yeasts either alone or with bacterin, onto glass slides inscribed with eight mm rings. The suspensions were allowed to dry in a forced warm air stream. The slides were heat-fixed and stained using Methylene Blue (MB), Giemsa, Diff-Quik, Gram, and Wright's reagents. Slides were examined by light microscopy at both 40x and 100x (oil) magnifications. Micrographs were captured using a 1.4 megapixel CCD USB 2.0 Camera (Lumenera Corporation, Ottawa, ON, Canada). Selected descriptions are provided below.

Selected microscopic results. Active yeast stained with MB indicated it is composed of individual cells, pairs (budding forms) and clusters containing about ten cells. It was apparent that the cell walls of a minority of active yeast were "incomplete" as suggested by their lighter color. Rarely bacteria were seen in these preparations.

Wright's stained Celmanax yeast was composed of a mixture containing heterogeneous cell shapes some of which resembled the budding forms of active yeast; others were elongated and spindle shaped. The staining intensity of cell walls varied form light to heavy. A variety of bacteria could also be seen most notably a very long bacillus type.

Gram stained mixtures of *Ra* serotype 1 bacterin with Celmanax Liquid showed areas where the yeast and the *Ra* were in close proximity. In some cases, it appeared that a bacterin coating enshrouded individual yeast cells. In other cases, where the bacterin existed in large multicellular clumps, it appeared as if the yeast and bacterin were intimate. In such cases, it appeared the more Gram negative yeasts were preferred and were able to make direct contact with the bacterin.

DISCUSSION

The data indicate that Celmanax a hydrolyzed yeast product designed for inclusion in animal feeds has a capacity to react with *Riemerella anatipestifer* the etiologic agent of serositis or duck septicemia, an important cause of morbidity and mortality in commercial ducks. The reactivity appears to include the capacity to agglutinate the bacteria. This property extended to both live *Ra* and some bacterins made by formalin inactivation. Reactivity with Ra was serotype dependent, however. Celmanax was more reactive with *Ra1* than with other serotypes. Microscopic examination of Celmanax indicated that the product was composed of yeast cells showing varying degrees of cell wall alteration presumably indicating hydrolysis. This characteristic was apparent using several staining reagents including the Gram method. Moreover, it appears that more highly hydrolyzed yeast cells are better able to be intimate with *Ra* bacterin. Field trials using Celmanax supplemented drinking water are currently in progress. These are located at a facility with a history of the disease of interest. Performance, morbidity and mortality data are being collected in order to determine the utility of this product in commercial duck production.

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Table 1. Yeast product.

Ra bacterin	"B"	"F"	Cmx Sol	Cmx Liq	Cmx Carrier	Antigen	Sera
1	+/- [A]	++	++	+++	+/-	-	++++
2 Ind	-	-	-	++	-	-	-
2 Wis	-	-	-	-	-	-	-
5	F	FF	FF	+F	F	f	++
6	-	-	-	-	-	-	+/-
1,2,5	-	+	+	?	-	-	+
Yeast Alone	-	+/-	-	+	-		
Yeast + Serum	-	++	+/-	+	-		

[A] +, ++, = degree of typical agglutination, f, F = "floc" type reactions

EVALUATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF STRAINS OF *MYCOPLASMA SYNOVIAE* ISOLATED IN THE ITALIAN POULTRY INDUSTRY

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ABSTRACT

Minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic able to inhibit the visible growth or metabolism of a microorganism in *in vitro* cultivation. Mycoplasmas are small prokaryotes lacking in cell wall. They are considered fastidious organisms because of their complex growth requirements. As mycoplasmas are responsible for severe economic losses in poultry industry, they are considered important pathogens.

For practitioners, the choice of a successful drug treatment could reduce the impact of several production problems. Even if antibiotics are licensed for a specific use, therapeutic success is not always guaranteed; therefore the knowledge of drug susceptibility on circulating mycoplasma strains provides additional information for antibiotic selection.

The aim of this study is to evaluate the antibiotic susceptibility of recent *Mycoplasma synoviae* strains isolated in different poultry commercial categories in order to provide a management tool for the poultry industry.

INTRODUCTION

Mycoplasma synoviae (MS) is considered an important pathogen for poultry. MS is associated with respiratory and articular disease, loss of production in layers and in meat sector, resulting in high rate of carcass condemnations. Recently, MS has been related to an unusual lesion of eggshell apex, classified as eggshell apex abnormalities (EAA) (1-3). In poultry sector, mycoplasmosis control is based on the production and maintenance of PPLO-like free breeders in association with high levels of biosecurity measures applied in the flocks. Even if this type of approach has determined the decrease of MS prevalence in the poultry industry, every year new MS outbreaks are described. In these cases the antibiotic treatment represents the only chance to contain the infection and consequently the economic losses. For these reasons and in order to give the practitioners a valid tool for the drugs choice, we decided to test the minimum inhibitory concentration (MIC) of 16 MS strains, isolated in the last two years from different production sectors (broiler, layer hen, chicken breeder, meat turkey, and guinea fowl).

MATERIALS AND METHODS

The 16 MS strains were harvested in our strains bank. The selection criteria applied were based on year of isolation (2009-2011), the sector of provenience, and genetic features related to *VlhA* gene as described by Hammond *et al.* (4). PCR products were processed for sequencing and the sequences were aligned and compared.

Propagation of strains and MIC tests were performed using the Mycoplasma Experience® Media without inhibitors. The MIC's procedure was carried out following the guidelines of Hannan (5) with slight

modifications. Antimicrobial agents are commercially available in microtiter plates (Sensititre®). Plates were incubated at 37 ± 1°C. Each strain was tested in duplicate and the reference strain (WVU 1853) was included in the study. Each plate contained a positive and negative control well. Antibiotics and their relative concentrations are shown in Table 1. MIC plates reading was done 24 to 48 h after the inocula, when the positive control well showed a typical acidification, revealing the mycoplasma growth. MIC breakpoints used in this study are reported in Table 2 (5-7).

RESULTS AND DISCUSSION

In all strains tested the positive control well showed an evident growth after 24 to 48 h of incubation whereas no growth was observed for negative control well. According to our procedure, the reading of plates was performed when the positive control well showed the growth. The MIC values are the lowest concentrations (µg/mL) where no mycoplasma growth occurred. Moreover we calculated the MIC 50 (i.e. the MIC able to inhibit the 50% of the strains tested) and MIC 90 (i.e. the MIC able to inhibit the 90% of the strains tested). Results are reported in Table 3.

Our strains resulted susceptible to tylosin, tilmicosin, lincomycin, and oxytetracycline. Antibiotic resistance was observed for erythromycin and enrofloxacin.

Studies focused on antimicrobial susceptibility of MS field strains are still scarce, despite the fact that some European reports highlight a decrease of MS susceptibility to fluoroquinolone drugs (2-8) and recommend that their use for MS disease treatment be avoided. Moreover we noticed that strains with higher MIC value for tylosin showed also higher MIC value for tilmicosin. These results could suggest the development of a common mechanism of drug resistance for this class of antibiotics. Finally, regarding the difference between MIC50 and MIC 90 it is possible to notice that some strains, even if considered still susceptible, showed a shift versus the intermediate breakpoint suggesting the development of antibiotic resistance mechanism.

The MIC study of MS field strains or of any other bacterial species could provide a useful tool choice for management of therapy in affected flocks. Moreover MIC gives the unique opportunity to establish if any antibiotic resistance mechanism is going to develop. This information is essential for a conscious use of the drugs in order to contain the drug resistance development and a successful recovery of animals.

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Table 1. Antibiotics tested and their relative concentration.

	Tyl	Tilm	Lin	Ery	Ox	Enro
A	0.12	0.12	0.12	0.12	0.12	0.12
B	0.25	0.25	0.25	0.25	0.25	0.25
C	0.5	0.5	0.5	0.5	0.5	0.5
D	1	1	1	1	1	1
F	2	2	2	2	2	2
G	8	8	8	8	8	8
H	32	32	32	32	32	32

Legend: Tyl: Tylosin; Tilm: Tilmicosin; Lin: Lincomycin; Ery. Erythromycin; Ox: Oxytetracycline; Enro: Enrofloxacin;
Minimum inhibitory concentrations are given in µg/mL.

Table 2. Drugs and relative breakpoints used in this study.

Antimicrobial agent	Breakpoint (µg-mL-1)		
	Sensitive	Intermediate	Resistant
Tylosin (Hannan *et al*., 1997)	≤1	≤ 2	≥ 4
Tilmicosin According to NCCLS standard M31-A2	≤ 8	16	≥ 32
Lincomycin (Kempf *et al*., 1989)	≤ 2-8		> 8
Erithromycin (Kempf *et al*., 1989)	≤1	≤ 4	> 4
Oxytetracycline (Hannan *et al*., 1997)	≤ 4	8	≥ 16
Enrofloxacin (Hannan *et al*., 1997)	<0.5	1	≥ 2

Table 3. MIC50 and MIC 90 for the tested strains.

	Tyl	Tilm	Oxy	Linc
MIC50	0,12	0,25	1	1
MIC90	0,50	8	2	1
Breakpoint	≤1	≤8	≤ 4	≤ 2-8

RICE HULLS AS POSSIBLE SOURCE OF *CLOSTRIDIUM BOTULINUM* TYPE C SPORES

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SUMMARY

In the last two years eight outbreaks of type C botulism have been signalled in farmed meat chickens and one in turkeys in the North of Italy. The diagnosis was obtained on the basis of clinical findings, mouse bioassay, PCR results, and *C. botulinum* type C isolation. Rice hulls were used as litter component in both turkey and in two chicken outbreaks.

Feed, water, and rice hull samples were collected in two affected broiler farms and analyzed as possible source of *C. botulinum* spores or neurotoxins. *C. botulinum* type C was detected by PCR in both batches of rice hulls stored before the use and employed as litter in affected farms. Moreover the strain was isolated from one positive rice hulls batch. Six samples of rice hulls collected from batches not related with botulism outbreaks tested negative for *C. botulinum* by PCR.

Results demonstrated that rice hulls can be a source of *C. botulinum* type C spores and a careful use of this material as litter for poultry should be considered.

INTRODUCTION

Botulism is a neuroparalytic disease caused by the action of the botulinal neurotoxin (BoNT) produced by the anaerobe spore forming bacillus *Clostridium botulinum*. BoNTs are classified into seven serotypes, A through G, on the basis of their antigenic properties (8).

C. botulinum type C is the most common serotype involved in avian botulism outbreaks although types A, D, and E botulism have rarely been reported in birds (6). BoNT type C and D encoding genes are carried by bacteriophages that are frequently lost during cultivation, making difficult the isolation of the strain (4, 5, 9)

Botulism may occur following the ingestion of preformed BoNTs (intoxication) or as the consequence of *C. botulinum* overgrowth and BoNTs production in the intestine or in a wound (toxico-infection) (6, 11, 12). In poultry the intoxication form has been demonstrated only in one outbreak where high titers of BoNTs type C have been detected in decomposing cannibalized chicken carcasses (1). In most cases the toxico-infectious form has been suspected even if spore source has rarely been detected (10).

In this report the results of the epidemiological investigations conducted in two broiler and one turkey Italian botulism outbreaks are presented.

MATERIALS AND METHODS

Case history. During the winter 2009-2010, two different farms located in Northern Italy, containing 120,000 and 46,000 broiler chickens, aged from 20 to 46 d experienced recurrent botulism outbreaks. Classic symptoms such as flaccid paralysis of the wings, legs, neck, and eyelids were recorded. Mortality rate ranged from two to five percent. Diagnosis was based on mouse test from sera of symptomatic birds and on *C. botulinum* type C detection by PCR and isolation from the intestinal contents of death birds. Mortality rate was contained by means of ampicillin administration in water (30 mg/kg).

In July 2011 two turkey flocks composed of 96,000 female and 46,000 males experienced botulism. Mortality rate was 17% in males and six percent in females. Rice hulls were used as litter component in both turkey and in the two chicken investigated outbreaks.

Epidemiological investigation. Food and water samples were collected in both chicken and turkeys outbreaks and tested for BoNTs and *C. botulinum* type C by mouse test and PCR respectively (2)(3). At the time of food and water sampling, unused amounts of the same rice hull batches employed in affected chicken flocks were collected and tested for BoNTs and *C. botulinum* type C. Rice hulls were washed in physiological solution (2:1) for five minutes using a stomacher (PBI international, Milan). Washing solution was filtered by sterile gauze and centrifuged (3000 X g) for ten min at 4°C. Supernatant was subjected to mouse test while one g of the sediment was introduced in nine mL of prereduced Fortified Cooked Meat Medium (FCMM) (7). FCMM tubes

were afterwards heat shocked in bath at 71 °C for ten min and incubated 48 h at 37°C. One hundred seventy-five µL collected from the bottom of the FCMM cultural broths were analyzed for *C. botulinum* type C by PCR (3). PCR positive FCMM broths were subjected to mouse test and subcultured for *C. botulinum* in agarized media as previously described (7). Suspected *C. botulinum* isolated colonies (lipase and lecitinase positive in egg yolk agar) were picked up, cultured in FCMM, identified by PCR, and filtered (0.45 µm) supernatant was tested by mouse test for BoNT production (2). Six batches of rice hulls sampled in different farms in which flocks did not experience botulism were analyzed for *C. botulinum* type C as described above. In turkey outbreak, stored rice hulls were not available and litter samples were collected in the sheds and analyzed as described above for rice hulls.

RESULTS

Feed and water samples tested negative for both BoNTs and *C. botulinum* spores. *C. botulinum* type C was detected by PCR in all rice hull samples collected in affected farms but not in samples collected in healthy flocks. Furthermore *C. botulinum* type C was also isolated in one rice hull PCR positive sample. Litter samples of affected turkey flocks tested positive for *C. botulinum* type C by PCR.

DISCUSSION

Several botulism outbreaks in broiler chickens have been reported in literature but the origin of the disease has remained often unexplained. Intoxication and toxico-infection forms have been proved to occur in chicken from cannibalized carcasses and contaminated food respectively (1, 10).

In the present investigations, rice hull batches used in affected flocks showed to be contaminated by *C. botulinum* type C spores. The hypothesis that a toxico-infectious form occurred is supported by the fact that preformed BoNTs have not been detected in possible sources and that ampicillin administration caused a decrease of morbidity and mortality. The significance of this finding is emphasized by negative results obtained from rice hull samples collected in healthy flocks, even if the number of tested batches should be increased.

Rice hulls (or rice husks) are the coating for the seeds of the rice plant and its contamination could have happened at harvest time, during the processing, or after its storage in the farm. Rice fields are often populated by waterfowl and some carcasses could accidentally be minced by threshing machine during harvest operations or in some following processing stages.

Similar pathogenesis have been observed in most bovine botulism outbreaks when *C. botulinum* spores are carried by carcasses accidentally incorporated in food and their germination, proliferation and BoNT production causes the toxico-infection form.

Results demonstrated that rice hulls can be a source of *C. botulinum* type C spores and a careful use of this material as litter for poultry should be considered.

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CLOSTRIDIUM SEPTICUM ISOLATES FROM CELLULITIS CASES IN TURKEYS APPEARS TO BE HIGHLY CONSERVED

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Cellulitis has emerged as an economically significant disease syndrome in turkeys in the United States. Mortality, increased condemnation rates, as well as expensive medication costs for treatment have accounted for the huge economic loss (1). Cellulitis is associated with acute mortality and presence of inflammatory subcutaneous fluid and crepitus, most commonly in commercial male turkeys nearing market age. The mortality is reported to be as high as 2-3 % per week in the affected flocks (2).

The lesions have been seen in various areas of the body, including: the breast, abdomen, legs, thighs, groin, and the back of the bird. Interestingly, in most cases of cellulitis, there appears to be no trauma to the skin. Palpation of the affected areas often reveals crepitation due to gas bubbles in the subcutis and musculature. At necropsy, there is accumulation of large quantities of bubbly, serosanguinous fluid in the subcutis (3). *Clostridium septicum* is considered an important causative agent of cellulitis in turkeys along with *Clostridium perfringens*. Use of a *Clostridium septicum* toxoid appears to be protective against cellulitis in turkeys in our experimental studies (4).

It is important that we examine the diversity of *C. septicum* isolates causing cellulitis in turkeys. The objective of our study was to examine the phylogenetic relationship of *C. septicum* isolates and also to identify their toxin profiles. In brief, *C. septicum* isolates obtained from cellulitis lesions in turkeys from Minnesota were included in this study. They were grown in brain heart infusion media. The DNA was extracted and was subjected to multi locus sequence typing (MLST).The culture supernatant from these isolates was subjected to SDS-PAGE analysis and two-dimensional gel electrophoresis to separate the proteins. The secretory toxin components were identified by MALDI-TOF mass spectrometry.

MLST analysis of *C. septicum* isolates yielded only two sequence types (ST). All the *C. septicum* isolates examined fell into a single cluster along with ATCC strain 12464 except UMNCS9 isolate. Two polymorphic sites were identified in the amplified fragment of gyrA. We observed only one type of proteomic profile for *C. septicum* isolates. The major secretory toxins we identified in *C. septicum* isolates were alpha toxin, septicolysin, sialidase, Dnase, transferrin, flagellin, and Gelsolin precursor (actin depolymerizing factor). Our results identified *Clostridium septicum* isolates belonging to two different sequence types but with similar protein profiles. When compared with *C perfringens, C septicum* isolates obtained from cellulitis cases appeared more clonal. The diversity observed among isolates of *C. septicum* in this study is considerably less than has been reported for similar analyses performed on *C. septicum* (5).

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GENETIC COMPARISON OF *CLOSTRIDIUM BOTULINUM* STRAINS ISOLATED FROM FARMED AND WILD BIRDS

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SUMMARY

Eighteen *Clostridium botulinum* type C strains isolated from wild birds (ducks and goose), farmed broiler chickens, rice hulls, and mammals have been genetically sub-typed and compared by means of random amplified polymorphic DNA (RAPD) analysis. Nine clusters with similarity higher than 80% have been obtained. Inconstant genetic connections have been observed among strains isolated in the same botulism outbreak from different subjects. Poor similarity (60.7%) has been observed between strains isolated from affected chickens and rice hulls, suspected as the source of the disease. The single neurotoxic gene transmission phages, mediated from rice hull strains to intestinal strains, have been hypothesized.

INTRODUCTION

Clostridium botulinum is a spore forming Gram positive, anaerobe obligate bacillus that produces a neurotoxin (BoNT) considered the most poisonous biological substance known (10). *C. botulinum* is classified in seven serotypes, A through G, on the basis of the antigenic properties of the BoNT produced. Type C is undoubtedly the most frequent serotype involved in avian botulism outbreaks even if type A, D and E have also been sporadically signaled in birds (8).

Type A, B, E and F BoNT encoding genes can be located at chromosomal level or carried by plasmids whereas, C and D BoNT encoding genes are carried by bacteriophages. Those phages are frequently lost during cultivation and this makes the isolation of *C. botulinum* type C and D difficult (6, 7, 12). The distinction between type C and D is not absolute because chimerical sequences composed by parts of BoNT/C and BoNT/D genes exist. The chimerical toxins showed to be more lethal to avian species than either type C or D (15).

In the last four years, several chicken botulism outbreaks have been reported in France, Sweden, Germany, and Italy (1,2,13,14). Not all outbreaks of the neurotoxin/spore sources have been investigated.

The recent development in molecular epidemiology has drawn attention to subtyping techniques in tracing and tracking pathogens, especially for *Clostridium botulinum*. Pulsed field gel electrophoresis (PFGE) is considered the gold standard for subtyping this microorganism but some strains can result untypeable by means of this technique due to the presence of extracellular DNAses and GC DNA methylation. Skarin *et al*. (13) showed that random amplified polymorphic DNA (RAPD) analysis can also be adopted to study possible relatedness between *C. botulinum* type C strains isolated in avian outbreaks.

The aim of this study was to compare RAPD profiles of *C. botulinum* type C isolated in farmed and wild birds.

MATERIALS AND METHODS

Strain collection. Eighteen strains isolated from symptomatic or dead animals in eleven different type C botulism outbreaks have been included in the study (Table 1). The disease was diagnosed on the basis of clinical signs, mouse test from sera or biological samples, PCR results and *C. botulinum* type C isolation. All the strains were isolated in egg yolk agar after a pre-enrichment procedure in Fortified Cooked Meat Medium (9) and each pure culture was identified by PCR (5), tested for toxicity by mouse test (11), frozen in cryogenic vials and stored at -80°C.

RAPD analysis. DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for Gram positive bacteria. DNAs were subsequently purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and RAPD analysis was performed using Illustra Ready-To-Go RAPD Analysis Kit (GE Health care) according to Skarin *et al*., (14). Amplification products were detected by means of gel electrophoresis on three percent of high resolution agarose gel (MethaPhor Agarose, Lonza) added with ethidium bromide. Evaluation of RAPD profile was performed using InfoQuest FP v.5 software (Bio-Rad). A UPGMA tree was constructed

using Dice similarity indices and optimization and position tolerance of 1.5%

RESULTS

Fragment patterns have been generated with all the six primers included in the RAPD kit used. Primer "two" showed to be the most discriminative. Patterns of the tested strains are displayed in Figure 1.

Tested strains can be grouped into nine clusters based on a similarity cutoff value of 80%. Strains isolated from ducks are randomly distributed in five different profiles without apparent similarities, neither for strains isolated from two different subjects death in the same outbreak (strains 4732/2/10 and 5009/2/10). Interestingly, the strain isolated from the coypu shows the highest similarity (67.5%) with the duck strain isolated in the same outbreak. Three strains isolated from the same rice hulls sample, showed the same RAPD profile each other and 77% similarity with a duck strain. Moreover they exhibited 60.7% similarity with chicken strains isolated in the same outbreak.

In the two broiler outbreaks different results have been achieved by RAPD analysis. In the first one all the isolates gave identical profiles (8103/09), but in the second outbreak the strains isolated from two different chickens (2659/1/10; 2659/2/10) showed 69.7% similarity.

The bovine strain has exhibited the lowest percentage of similarity (43.9%) with the other tested *C. botulinum* type C.

DISCUSSION

RAPD analysis is a rapid, ready to use PCR sub-typing method that has been applied to different bacterial species, *C. botulinum* type C included. Previously results demonstrated that PFGE and RAPD profiles overlapped and that primer "two" of RAPD kit used in this same study is the most discriminative one (14). Our results confirmed the discriminative power of primer "two" but difficulties in the image elaboration due to many weak bands were raised.

Low genetic relatedness has been observed between strains even if isolated in the same outbreak from different subjects. This result has been observed both in ducks and in chicken outbreaks. On the contrary, in Swedish epidemic outbreaks, all the strains of chicken origin isolated in different farms showed the same PFGE and RAPD profile.

In the bacteriological examination *C. botulinum* type C colonies are suspected on the basis of a few biochemical characteristics that are common to other clostridia (e.g. lecitinase, lipase). The definitive identification is achieved by the mouse test (toxic effect neutralization with specific antitoxins) or by PCR protocols, targeted on the neurotoxin gene. However it has been demonstrated that phages carrying C or D encoding genes can be harbored by other bacterial species and that *C. botulinum* type C can be infected by phages from type D and *C. novyi* type A (3, 4). For the above reasons it is possible to hypothesize that not only *C. botulinum* spores can be ingested and overgrow in the intestine causing the disease, but also that phages can switch from the progenitor strain and transfer toxigenic genes to other autochthonous gut microorganisms. In that last occurrence, strain subtyping methods based on the whole genome could be inadequate for epidemiological investigations. Furthermore this could explain the genetic differences observed between rice hulls strains and chicken strains by RAPD analysis. However this hypothesis needs to be demonstrated by means of sub-typing methods based on the BoNT gene characterization.

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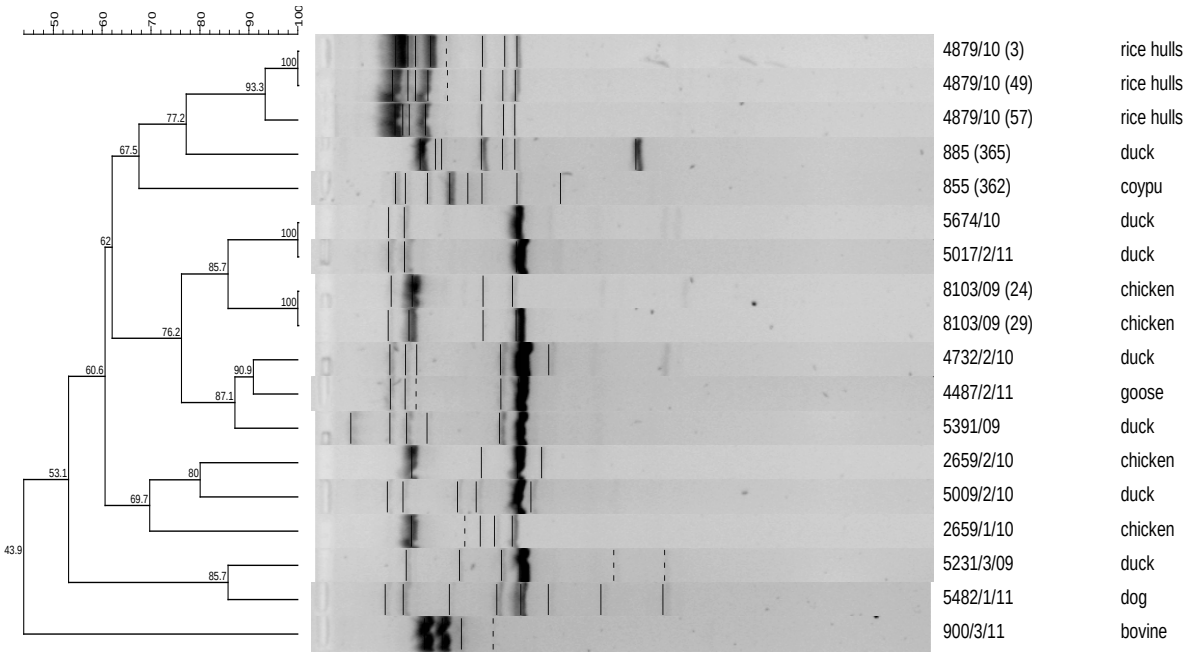
Table 1. Strains included in the study.

ID strain	Species	ID Outbreak	Organ/Material
885 (365)	Duck	1	liver
855 (362)	Coypu	1	intestine
5674/10	Duck	2	intestine
5017/2	Duck	3	intestine
4732/2/10	Duck	4	intestine
5009/2/10	Duck	4	intestine
5391/09	Duck	5	intestine
5231/3/09	Duck	6	intestine
8103 (24)	Chicken	7	intestine
8103 (29)	Chicken	7	intestine
2659/1/10	Chicken	8	intestine
2659/2/10	Chicken	8	intestine
4879 (3)	Chicken	8	rice hulls
4979 (49)	Chicken	8	rice hulls
4979 (57)	Chicken	8	rice hulls
4487/2	Goose	9	intestine
5482/1	Dog	10	feces
900/3	Bovine	11	liver

Figure1. RAPD profiles of tested strains.



STUDIES ON PERITONITIS IN EGG-LAYING CHICKENS POST EXPERIMENTAL INFECTION WITH *ORNITHOBACTERIUM RHINOTRACHEALE* ALONE AND ALONG WITH OTHER AGENTS

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INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a respiratory pathogen affecting turkeys, chickens, and wild birds. ORT in chickens has resulted in economic losses to the poultry industry. In chickens, respiratory lesions include airsaculitis and pneumonia. The clinical disease post experimental ORT infection in broiler chickens appears to be much less severe than in turkeys and is limited to sneezing, rhinitis, and growth retardation without causing any macroscopic lesions. However, co-infection with infectious bronchitis virus (IBV) has resulted in airsacculitis. In turkeys ORT infection show depression, coughing, bloody nasal discharge, tracheitis, airsacculitis, fibrinous pneumonia and pericarditis, and mortality.

Leghorn chickens have been reported to be least susceptible to ORT infection as compared to broiler chickens and turkeys. Though the clinical disease appears less severe in chickens, seroprevalence of ORT infection has been reported to be high in many broiler breeder flocks and in layer flocks in north central regions of United States (Heeder etal, 2001).

The objective of this study was to investigate the role of ORT alone and/or with IBV and *Escherichia coli* on peritonitis in egg-laying chickens.

MATERIALS AND METHODS

ORT serotype C and *E. coli* serotype O78 of chicken origin were used in this study. Arkansas strain of IBV was used for concurrent infections.

One hundred and sixty, 80 wk old SPF White Leghorn laying hens were used in this study. The birds were kept in individual layer cages on layer feed and water *ad libitum*. All the birds were negative to antibodies to ORT by the serum plate agglutination test and ELISA.

The birds were randomly divided into groups I through VIII containing twenty birds each. Birds in

groups III, V, VI and VII were inoculated intra-tracheally with one mL of 1x 10^6 mean EID_{50} of IBV per bird. Birds in groups II, IV, VI and VII were aerosolized with one L each of $2x10^8$ CFU/mL of *E.coli* serotype O78 using a power sprayer. On d five post IBV/*E.coli* infection, birds in groups I, IV, V and VII were inoculated intra-tracheally with one mL of 10^8 CFU of ORT per bird, made in sterile phosphate buffer saline (PBS). Birds in group VIII were inoculated intra-tracheally with sterile PBS and used as sham inoculated controls.

All the birds were then monitored daily for any clinical signs or mortality. Two birds from each group were euthanized on 2, 4, 7, 14, 21, and 28 d post-ORT-infection and necropsy was conducted. Euthanasia was performed in a carbon dioxide chamber. Gross lesions were recorded in each bird. Tissues were collected in sterile Whirl-pak filter bags for bacteriologic examination or in 10% buffered neutral formalin for immunohistochemistry and histopathology. Samples of trachea, lungs, liver, spleen, kidney, oviduct, and bursa were homogenized and inoculated on SBA and MacConkey agar for isolation and identification of ORT and *E.coli*, respectively. In addition, sterile swabs were collected in PBS from air sac and peritoneum for isolation and identification of ORT and *E.coli*. After 24 h, suspected colonies were sub-cultured and tested for oxidase and catalase activity. Samples of trachea and lungs were homogenized in PBS containing penicillin-streptomycin and egg-inoculated for isolation of IBV. Infectious bronchitis virus was later confirmed by RT-PCR.

Blood samples were collected from treatment and control groups at 0, 7, 14, and 21 d post-ORT infection. The sera were examined for the presence of antibodies against ORT by ELISA. The hematoxylin and eosin (H&E) staining technique was used for histopathological examination. A scoring system (lesion score varied from one to four depending on the increased severity of the lesions) was used to evaluate histopathological changes in different tissues for analysis and comparison. Trachea, lungs, liver, spleen, kidney, and oviduct were subjected to immunohistochemistry for detecting ORT antigens. One way analysis of variance was employed for statistical analysis of the data. A *P* value of 0.05 was considered as significant.

RESULTS AND DISCUSSION

Birds infected with IBV alone showed ruffled feathers and droopiness whereas birds infected with IBV + *E .coli* showed severe nasal discharge and were off feed. Two birds in group VII (IBV + *E. coli* + ORT) and three birds in group VI (IBV + *E. coli*) succumbed to death on two d post-IBV + *E. coli* infection. Following ORT infection, no mortality was noticed in any groups until 16 d post ORT infection. In the IBV + *E. coli* + ORT infected group, one bird succumbed to death on 16 d post-ORT infection. Although egg production dropped in the ORT + *E. coli* alone infected groups when compared with non-infected control birds in group VIII, an abrupt cessation of egg production was noticed in all the birds in the IBV infected groups.

Airsacculitis, lung congestion, and pericarditis were noticed in most of the birds infected with IBV + *E. coli* and IBV + *E. coli* + ORT on two and four d post- ORT infection during necropsy. Peritonitis was noticed in two birds necropsied from the IBV + *E. coli* + ORT infected group on seven and 14 d post-ORT infection. One bird from this group, which died on 16 d post-ORT infection showed pericarditis, perihepatitis and peritonitis. In addition, an accumulation of amber colored fluid, the presence of cheesy deposits and thickening of visceral peritoneum was noticed in all the three affected birds. No gross pathological changes were noticed in any of the tissues in birds infected with ORT or *E. coli* alone.

In birds of group I, ORT was isolated from sinus, trachea, airsacs, liver, and kidney. In groups IV, V, and VII, ORT was isolated from all the above organs as well as from lungs and oviduct. We isolated ORT from air sacs and trachea even on 14 d post-ORT infection and from sinuses up to 28 d post-ORT infection IBV + *E. coli* + ORT infected group. In 20-50% of all the ORT infected birds we were able to isolate ORT from the infraorbital sinuses on 28 d post-ORT infection. From birds in groups IV, VI and VII, *E. coli* was isolated from the sinuses and trachea on d two and four post-ORT-infection. Both ORT and *E. coli* were isolated from the peritoneal swabs collected from all the three birds having peritonitis.

Histopathological examination in the tracheal tissues of birds in group I to VII showed infiltration of lymphocytes, plasma cells, and macrophages in the lamina propria. Lymphoid aggregates were present in severely affected tracheas which caused the mucosa to bulge into the lumen. A few of the glands were dilated and empty and the cilia were disrupted. There were moderate numbers of necrotic cells in the BALT. There were focal areas of fibrin exudation and infiltration of inflammatory cells in the lungs in birds infected with IBV+ ORT and IBV + *E.coli*+ ORT. Liver sections showed infiltration of portal tracts by varying number of lymphocytes and macrophages and fibrin deposition.

The peak histopathologic lesion score was obtained on fourth d post-ORT infection. The overall histopathologic lesion score observed following ORT infection alone (Group I) was found to be statistically significant ($P<0.05$) when compared with the control group (group VIII). The histopathologic lesion score

was numerically higher in ORT groups co-infected with IBV and *E. coli* (groups IV and V) but was not statistically significant (*P>0.05*) when compared with the lesion scores in ORT alone infected groups. However, the overall histopathologic lesion score was significantly higher (*P<0.001*) for the group infected with ORT + IBV + *E. coli* when compared with any other groups.

All birds infected with ORT became seropositive to ORT by ELISA on d seven of ORT infection. The ORT antibody titers remained high even on d 28 post-ORT infection. Peritonitis was noticed in only two birds necropsied from the IBV + *E. coli* + ORT infected group on seven and 14 d post-ORT infection. The results appear to indicate that ORT alone may not play any significant role in peritonitis in egglaying chickens unless there are other complicating concurrent infections.

DIFFERENTIATION OF PATHOGENIC AVIAN MYCOPLASMAS BY REAL-TIME POLYMERASE CHAIN REACTION

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SUMMARY

The pathogenic avian mycoplasmas are associated with respiratory disease, synovitis, and decreased production performance. IDEXX Laboratories has developed species-specific, real-time polymerase chain reaction (real time-PCR) reagents. These assays detect specific sequences from purified DNA samples and all share cycling conditions to allow for simultaneous and accurate high through-put testing.

INTRODUCTION

Pathogenic *Mycoplasma* spp. are widely prevalent, and detrimental to poultry health and production. Three common species are *M. gallisepticum* (Mg) and *M. synoviae* (Ms), found in chickens and turkeys, and *M. meleagridis (*Mm*)*, found in turkeys. Infection with these pathogens can lead to airsacculitis, synovitis, respiratory disease, decreased growth and decreased egg production (3). Detection has historically been via hemagglutination inhibition (HI), serum plate agglutination (SPA) and enzyme linked immunosorbance assay (ELISA), with culturing as the gold standard of testing. The use of real-time PCR for the detection of pathogenic mycoplasmas has emerged as a rapid and highly accurate technique for the recognition or confirmation of common *Mycoplasma* spp. in a flock. The real-time PCR reagent sets use hybridization probes to allow for the creation of melting curves that provide another level of diagnostic sensitivity and confidence in test results.

The samples used in our testing were DNA, extracted from a tracheal swab sample, using a commercially available extraction kit. Extraction procedure was done following package insert recommendations (6).

Three species specific, real time-PCR reagent sets were created to allow for the detection of the Mg, Ms and Mm DNA. Hybridization probes were chosen for use in the Detection Mix in order to simultaneously generate melting curve and melting temperatures (Tm). This allows for qualification and differentiation of the DNA segments of interest. The DNA sequences used to select the probes and primers were based on amplicon sequences reported by S. Kleven and Z. Raviv (2). The 16S-23S ribosomal intergenic spacer regions of Ms and Mm are used for those assays as it is a region that allows differentiation of the pathogenic mycoplasmas but is consistent within a species (4). A region of the mgc2 gene was chosen for use in the Mg assay as it has been shown to be conservative for Mg in other PCR protocols in the USA (5). These probes are recycled throughout the amplification process of the assay and cause fluorescence resonance energy transfer when both the donor and acceptor probes are bound to the target sequence. The light signals emitted are collected by the instrument used to run the real time-PCR and recorded to simultaneously capture crossing point value and produce the melting curve. This facilitates rapid diagnostic results.

An internal control was developed and included in the reagent set, adhering to recommendation by the OEI for PCR use for detection of mycoplasmas (6). Also, a weak positive control is included to assure fidelity of the results without the risk of overgrowth of the sample DNA.

The reaction mix used to run the PCR consists of four uL of Master Mix (Buffer, MgCl2, DNA polymerase, and dNTPs), two uL of Detection Mix (primers, probes, and internal control), nine uL of PCR grade water, and five uL of the extracted DNA sample

to be tested. This final mix is added to the wells to be tested and run on a cycling program based on 45 amplification cycles. This protocol was validated on a Roche Lightcycler 480 instrument with a 96 well block. The same cycling program is used for all three assays, allowing for simultaneous runs within one block.

MATERIALS AND METHODS

Test samples. The majority of samples used during validation consisted of purified DNA, extracted using a commercial extraction kit. A small group of initial tracheal swab samples were boiled for five min followed by a ten min centrifugation to pellet cell debris prior to addition to PCR reactions, with no specific DNA extraction performed. In cases where commercial extraction kits were utilized, the procedure was followed according to package insert recommendations (7). Samples were acquired from various farm sites.

Assay conditions. All samples were run according to reagent set instructions, using IDEXX Mg Detection Reagents (lot #1058), Ms Detection Reagents (lot #1060) or Mm Detection Reagents (lot #1062). Briefly, reactions consisted of four µL Roche Genotyping Master Mix, nine µL nuclease-free PCR grade water, two uL of species -specific detection reagent and five µL DNA extraction sample. Cycling conditions were as follows:

Activation:	95°C, 10 min, 1 cycle
Amplification (45 cycles):	95°C, 20 sec 60°C, 20 sec, single acquisitions 73°C, 15 sec 95°C, 1 min 45°C, 1 min
Melting Curve:	80°C, 0.14°C/sec ramp rate, continuous acquisitions

All samples were tested on a Roche LC 480 LightCycler at either IDEXX Laboratories or field sites, using 96-well reaction plates with optical film covers.

RESULTS

Analytical specificity. To determine the specificity of the IDEXX mycoplasma detection reagents, samples containing known *Mycoplasma* spp. were tested for the presence of Mg, Ms, or Mm DNA. As shown in Figure 1, none of the detection reagents were able to detect any of the foreign orthologous mycoplasma DNA samples. Results from these studies show the IDEXX detection reagents to be highly specific.

Diagnostic sensitivity and specificity. The sensitivity and specificity of the IDEXX detection reagents were determined by testing samples from multiple sites. All samples consisted of purified DNA, with the exception of one site in which a boiled sample was used, as described in the above section. All samples were tested using both IDEXX Detection Reagents and site laboratories standard real-time PCR testing protocol. Figures 2-4 summarize the results of all testing. As shown, all tests correctly identified samples containing mycoplasma DNA while maintaining high specificity with no false positive results.

DISCUSSION

In this work we present the results of analytical and clinical test results for IDEXX *Mycoplasma gallisepticum*, *M. synoviae*, and *M. meleagridis* detection reagents. Our results demonstrate these reagents to be both highly sensitive and specific for detection of mycoplasma DNA.

The primary goal was to develop standardized reagent sets for use in detecting pathogenic avian *Mycoplasma* spp. via real time-PCR. The objectives set forth in producing these products were to achieve high sensitivity and specificity with easy to use, uniform reagents across laboratories. Results suggest that these reagent sets will help improve consistency in testing results. To adhere to these goals, we chose the real time-PCR format, as it generated more rapid results than standard PCR. The use of fluorophore labeled hybridization probes allowed for simultaneous recognition of melting temperatures and the generation of melting curves.

Although some multiplex PCR products used for detection of pathogenic avian mycoplasmas have been described (5), we found that higher sensitivity could be achieved with a single target organism test. By running the different reagent sets on the same cycling program, users are able to run multiple real time-PCR tests at the same time, in different wells.

The internal control included with the reagents provides a safeguard to assure that there is no contamination by DNA amplification inhibitors present. There is a specific detection mix for the internal control included with the reagents that is composed of primers and probes specific for the internal control template.

CONCLUSION

The use of real time-PCR for the detection of pathogenic avian mycoplasmas can improve lab efficiency and provide economical and rapid results.

Our commercially available reagents, specific for detection of Mg, Ms, or Mm, provide excellent sensitivity and specificity when compared to standards of testing currently in place. The use of hybridization probe technology allows for the simultaneous generation of crossing point data as well as melting curve analysis to confirm positive results. We hope that these standardized reagent sets will improve testing confidence and be incorporated into current laboratory protocols as a robust and economical alternative to current methods of confirmatory testing.

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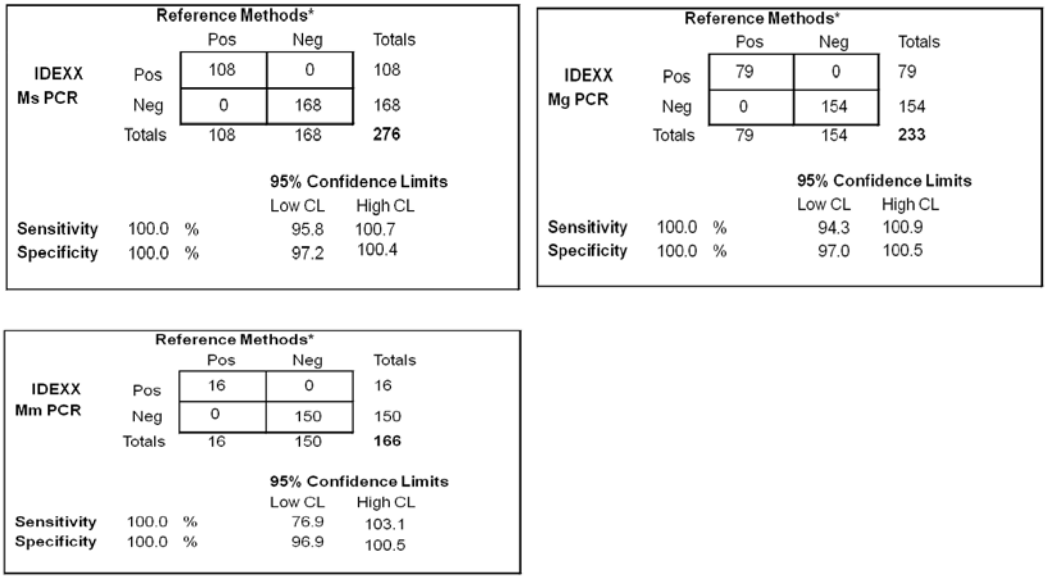
Figure 1. Analytical specificity for the IDEXX Mg, Ms, and Mm detection reagents.

Sample DNA	*M. gallisepticum*		*M. synoviae*		*M. meleagridis*	
	Crossing Point	T_m	Crossing Point	T_m	Crossing Point	T_m
M. iowae	negative	negative	negative	negative	negative	negative
M. lipofaciens	negative	negative	negative	negative	negative	negative
M. cloacale	negative	negative	negative	negative	negative	negative
M. imitans	negative	negative	negative	negative	negative	negative
M. gallisnarum	negative	negative	negative	negative	negative	negative
M. gallinaceum	negative	negative	negative	negative	negative	negative
M. glycophilum	negative	negative	negative	negative	negative	negative
M. pullorum	negative	negative	negative	negative	negative	negative
M. gallopavonis	negative	negative	negative	negative	negative	negative
10 fg	28.58	63.99	28.92	68.48	28.71	71.48
1 fg	31.70	63.64	32.22	67.92	32.84	71.42
nc	negative	negative	negative	negative	negative	negative
nc	negative	negative	negative	negative	negative	negative

Figures 2, 3, and 4.



Reference Methods*		Pos	Neg	Totals
IDEXX Ms PCR	Pos	108	0	108
	Neg	0	168	168
	Totals	108	168	276

		95% Confidence Limits	
		Low CL	High CL
Sensitivity	100.0 %	95.8	100.7
Specificity	100.0 %	97.2	100.4

Reference Methods*		Pos	Neg	Totals
IDEXX Mg PCR	Pos	79	0	79
	Neg	0	154	154
	Totals	79	154	233

		95% Confidence Limits	
		Low CL	High CL
Sensitivity	100.0 %	94.3	100.9
Specificity	100.0 %	97.0	100.5

Reference Methods*		Pos	Neg	Totals
IDEXX Mm PCR	Pos	16	0	16
	Neg	0	150	150
	Totals	16	150	166

		95% Confidence Limits	
		Low CL	High CL
Sensitivity	100.0 %	76.9	103.1
Specificity	100.0 %	96.9	100.5

*Reference methods: IDEXX reagents vs. internally designed PCR reagents and protocol from commercial labs in USA.

ALLTECH'S 37 + MYCOTOXIN PROGRAM: A TOOL FOR BETTER UNDERSTANDING MYCOTOXINS

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SUMMARY

Aflatoxin B1, ochratoxin A, vomitoxin (DON), T-2 toxin, and fumonisin B1 have been studied extensively for their presence in feed as well as for their effects on health and performance of poultry. These five mycotoxins, however, are only the marker for that particular group of mycotoxins and the concentrations listed against them do not include the presence of other mycotoxins in the same group. For example, the presence of DON in a poultry feed can indicate the presence of other DON-like mycotoxins such as 3-acetyl DON and 15-acetyl DON. If we do not analyze the feed for these two mycotoxins, the total impact of DON on animals may be under estimated. Alltech's 37+ Mycotoxin Program, wherein 37 different mycotoxins are analyzed using sophisticated LC-MS/MS instrument, have indicated the presence of many such lesser known mycotoxins. This program is allowing the better understanding of mycotoxin interactions within birds.

Around 500 mycotoxins have been characterized to date. However, only few have been tested in the field. In North America, deoxynivalenol (vomitoxin or DON) is widely tested with occasional testing of T-2 toxin, zearalenone (ZEA) and fumonisins. Thin layer chromatography (TLC) and enzyme linked immunosorbent assay (ELISA) are the common methods employed for these tests at feed mills and laboratories.

Since one mold can produce several mycotoxins and several molds can be present in one feedstuff, it is expected that there are likely more mycotoxins present than are being tested. To give an example, if a sample contains DON there are chances for the presence of several other mycotoxins including 3-acetyl DON, 15-acetyl DON, and fusarenon-X. As these toxins can contribute to the toxicity of DON, not analyzing them will give false sense of security. As a result, the ability to precisely analyze as many toxins as possible at an affordable cost and in a timely manner will assist in the better understanding of total toxicity to animals as well as developing preventive solutions (Berthiller et al.,

2005). The objectives of the current study, therefore, were to evaluate US feeds and feed ingredients for multiple mycotoxins using ultra performance liquid chromatography – two dimensional mass spectrometry (UPLC-MS/MS) technique.

MATERIALS AND METHODS

Mycotoxins tested. Although it is not practical to analyze all the known mycotoxins in the animal feeds and feed ingredients, 72 samples (most of them harvested in 2011) collected from different regions of US were subjected to the analysis of the following mycotoxins:

- **Aflatoxins:** B_1, B_2, G_1, and G_2
- **Ochratoxins:** A and B
- **Type B trichothecene mycotoxins:** DON, 3-acetyl DON, 15-acetyl DON, Nivalenol, Fusarenon-X, masked DON
- **Type A trichothecene mycotoxins:** T-2 toxin, DAS, HT-2 toxin, Neosolaniol
- **Fumonisins:** Fumonisin B_1, B_2 and B_3
- Zearalenone
- **Silage mycotoxins:** Patulin, roquefortine C, penicillic acid, gliotoxin, mycophenolic acid
- **Ergot mycotoxins:** 2-bromo-alpha-ergocryptine, ergocornine, ergometrine, ergotamine, lysergol, methylergonovine

In addition to 32 mycotoxins listed above, three isotopically labeled toxins and two other toxins, capable of qualitative analysis only at this stage, make up the total 37 mycotoxins tested. The major criteria for selecting these mycotoxins include their prevalence in the field as well as established animal toxicity.

Sample type. The major samples tested included corn, corn silage, haylage, barley, wheat, and oats. Limited numbers of poultry feed, TMR, wet brewers, high moisture corn, and beet pulp were also tested. For the ease of interpretation of the total toxicity to animals, the toxins of similar structure and effects were clubbed into groups as above.

RESULTS

Detection percentages. Only three samples out of 72 tested were negative for all the mycotoxins tested, giving 96% as level of contamination. Type B mycotoxins were detected in 83% of the samples followed by fumonisins (74%), ZEA (49%), aflatoxins (47%), and Type A mycotoxins (46%). Ergot toxins and ochratoxins were present in 29% and 22% of samples tested, respectively. Silage mycotoxins were present in 57% of the silage samples tested. The data shows clearly that unlike the 2009 harvest, the 2011 US harvest was contaminated with multiple mycotoxins. Although anecdotally it was believed that silages can be contaminated with mycotoxins, this field data provided a strong support.

Multiple mycotoxin occurrences. Only 5.6% of the samples tested contained one mycotoxin (Table 1). The highest percentage of samples, 34.7%, contained two to five mycotoxins followed by 27.8% and 12.5% of samples containing six to ten, and 16 to 20 mycotoxins, respectively. Five percent of the samples contained as high as 25 to 30 mycotoxins indicating the wide-spread occurrence of multiple mycotoxins in North American feeds and feed ingredients. Some of the corn samples contained as many as 18 different mycotoxins.

Ingredient contribution. Although molds can attack any feed ingredient, certain feed ingredients are known to contribute more towards some groups of mycotoxins (Table 2).

CONCLUSIONS

1) Use of Alltech's 37+ Mycotoxin Program allowed for better understanding of mycotoxin profile of North American feeds and feed ingredients.

2) Unlike 2009 crops, 2011 crops are contaminated with multiple mycotoxins and hence the increased risk due to mycotoxin interactions.

3) The implementation of Alltech's MIKO program (HACCP-like approach for mycotoxin control), on farms and feed mills enables the integrated approach to negate mycotoxin challenges.

4) The supplementation of feeds with a well researched, low level inclusion, and broad spectrum mycotoxin adsorbent can be one of the effective tools in mycotoxin management.

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Table 1. Percentage of total samples with multiple mycotoxins.

No. of mycotoxins in the same feedstuff	% samples
1	5.56
2 to 5	34.72
6 to 10	27.78
11 to 15	4.17
16 to 20	12.50
20 to 25	5.56
25 to 30	5.56

Table 2. Ingredient contribution to mycotoxins.

Ingredient	Mycotoxin Contribution
Corn	Fumonisins and Trichothecene B mycotoxins (eg. DON)
Corn Silage	Ergot toxins, Trichothecene B mycotoxins (eg. DON) and Fumonisins
Haylage	Ergot toxins, Trichothecene B mycotoxins (eg. DON), ZEA, Trichothecene A mycotoxins (T-2), Aflatoxins
Oats	ZEA, Trichothecene B mycotoxins (eg. DON), Ergot toxins, Trichothecene A mycotoxins (eg. T-2) and Fumonisins
Wheat	Trichothecene B mycotoxins (eg. DON), ZEA
Barley	Ergot toxins, Trichothecene B mycotoxins (eg. DON)

NATURAL ANTIBODY AND COMPLEMENT IN POULTS EXPOSED TO DIETARY DON AND LPS INJECTION

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SUMMARY

Serum from 26 d poults was tested by microtiter for natural antibody levels and complement. Agglutination and lysis titers to rabbit, horse and human erythrocytes were lowered by *E. coli* LPS injection. Inclusion of deoxynivalenol (DON) in the diet did not exacerbate the effects caused by lipopolysaccharide (LPS). The study of natural antibody and complement provide a convenient means to obtain information on both innate and acquired immunity in turkeys.

Fowl sera possess a broad spectrum of natural antibodies reactive with xenogenic erythrocytes (1, 4). Those recognizing rabbit erythrocytes (Rb) can agglutinate and lyse these targets and may be high titer. Others, reactive with human type O erythrocytes (HuO) and horse erythrocytes (Ho) are found, but typically at lower titers. These systems offer a practical advantage to investigators because they can be included as study components without the logistic complications accompanying artificial immunizations. Some of these antibodies are good agglutinins, activate complement, and so provide information on both innate and acquired immunity. Since they are quantified by classic techniques, measurement is not cumbersome. They were studied in sera obtained from tom turkey poults fed graded levels of deoxynivalenol (DON) and injected with *E. coli* lipopolysaccharide (LPS).

MATERIALS AND METHODS

Experimental birds. Nicholas male poults were raised in stainless brooder batteries. Temperature was 36.7°C during wk 1 and lowered weekly by 2.7°C. A standard turkey starter diet (2820 ME/kg) containing DON at 0.27 (basal), 2.49, 4.79, 7.57, and 10.24 mg/kg as determined by analysis was fed (10).

LPS. *E. coli* 055:B5, (Sigma-Aldrich), 1 mg/kg by i.p. injection on d 25; control poults were not injected.

Erythrocytes. Rb and Ho blood were purchased from a commercial supplier. HuO was donated by an adult, known to be type O. Erythrocytes were stored in Alsever's solution at 4°C.

Measurement of antibody and complement. The microtiter procedures were similar for each system. Fifty uL of poult serum was serially diluted with PBS supplemented with Mg (2.5 mM) in standard 96 well U bottom microtiter plates. Ten uL of 0.5 % (Ho) or 1% (Rb, HuO) washed erythrocytes was added to all wells, incubations were at room temperature. Agglutinins were assessed using HA1, HA2, and HA45 titers indicating reaction strength. L100 and L50, indicating complete or partial lysis, were used as complement (C') titers (3, 4). Titers measure the reaction end points, using the number of the highest well showing each response.

RESULTS

Sera were available from 36 LPS injected poults and 30 non-injected controls. Among the three types studied, Rb agglutinins and lysins were more commonly found and in the highest titer (Table 1). Rb agglutinins could be differentiated into HA1 and HA2 types in 21/66 (32%) poults. These indicate IgM and IgG isotype reactions, respectively. Both Ho and HuO agglutinins and lysins were also present but at lower titers. Agglutinin and lysin titers in LPS injected poults were typically lower than in control poults. Means tended to decrease with increasing DON levels but the trends were not statistically significant. Rb HA1, Rb HA2, and Rb L100, means of groups injected with LPS and fed DON were lower than those without LPS; but the reverse was true for Rb L50. None of the interactions were significant, however.

Ho agglutinins were present in all samples but they were of a peculiar type detected only after reducing the percentage of indicator cells from 1% to 0.5%. After doing so, stronger agglutinins were found in 46/66 (70%) of the poults and weaker agglutinins (HA45) were found in all poults. Differentiation of the stronger Ho agglutinins into HA1 and HA2 types could not be made with confidence, however. Ho lysins were found in 25 poults (38%) and tended to be lower with LPS treatment (log 2 means; LPS = 0.36, NoLPS = 0.70; Pr = 0.07).

HuO agglutinins were the least frequent and the lowest titer (log 2 means: LPS = 0.28, NoLPS = 0.33). Sixteen poults (24%) had agglutinins detectable without plate tilting, and all were positive for the weak (HA45) form detected by plate tilting (log 2 means: LPS = 3.08, NoLPS = 3.33, Pr = 0.32). Lysins were found in 15/66 (23%) poults (log 2 means: LPS = 0.25, NoLPS = 0.20, Pr = 0.64).

DISCUSSION

Natural antibody systems reactive with three xenogenic erythrocytes were present in the sera of 26 d poults. Rb agglutinins were in higher titer than either Ho or HuO. These results are not surprising given that the Rb agglutinins likely represent the "anti-Gal" system of this species (2). These arise because of gut microbial stimulus and so are similar in origin to human ABO antibody. The stimulating epitope is identified as a galactose disaccharide, Galα(1-3)Galβ(1-3)4GlcNAc-R, present on both microbes and Rb cells (6). The Ho and HuO stimulating epitopes are unknown, but likely of microbial origin, or possibly diet (9).

Each of these cells was capable of activating poult complement and lytic titers were higher with Rb. It is suggested that this is due to both alternate pathway (AP) and classic pathways (CP) (4). Thermal denaturation and chelation differences between activation by Rb and HuO suggest that the latter occurs by the AP (5). This is probably also the case for Ho lysis as well (7). These methods provide a convenient means to study immunity in poults.

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Table 1. Rb agglutinin and lysin titer means (log 2) of poults injected with LPS or not injected and fed diets containing graded levels of DON.

Treatment	**Rb HA1**	**Rb HA2**	**Rb L100**	**Rb L50**
LPS	0.92	1.00	2.03	3.53
NoLPS	1.43	1.80	2.63	4.30
Pr[A]	0.28	0.20	0.02	0.01
DON	**Rb HA1**	**Rb HA2**	**Rb L100**	**Rb L50**
0.3	1.33	1.40	2.53	4.00
2.5	1.00	1.38	2.15	3.92
4.8	0.92	1.15	2.46	4.00
7.6	1.27	1.53	2.27	3.87
10.2	1.20	1.30	2.00	3.50
Pr[B]	NS	NS	NS	NS

Pr = probability determined by [A]ANOVA or [B] regression analysis

CELLULAR IMMUNE RESPONSES IN TURKEYS INFECTED WITH *EIMERIA ADENOEIDES*

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INTRODUCTION

Coccidiosis is an important disease of turkeys caused by parasites of the genus *Eimeria*. One of the most common species that infects the turkey is *E. adenoeides* that develops in the cecum. Large doses of oocysts of this parasite can cause reduced feed intake, decreased weight gain, and high mortality in young poults (Clarkson, 1958). Infection with *Eimeria* is known to stimulate a protective immune response in chickens (Rose, 1996) but little is known concerning the development of protective immunity in species that infect the turkey and cellular immune responses to infection.

A series of experiments were conducted to investigate the development of immune responses to *E. adenoeides* infection in turkey poults. The first experiment was done with an objective of investigating the development of immunity and determining the alterations in blood leukocyte profiles following primary infection. The second experiment was conducted with an objective of investigating the local cellular immune activities occurring at the site of infection, the cecum.

MATERIALS AND METHODS

Experiment 1

The experiment comprised two phases – a primary infection and secondary infection.

Primary infection. Turkey poults at 20 d of age were allocated to two treatment groups, each comprising four cages of 12 birds per cage, and were reared in two separate test rooms in clean battery cages. They were each given a primary inoculum of 12.5 x 10^3 sporulated oocysts (room one, infected) or given a sham dose of water (room two, uninfected). Five d post-primary infection the birds were moved to clean battery cages every day until challenge in order to limit the secondary exposure of birds to oocysts produced and excreted in the feces. Weight gain was recorded from d zero through six of primary infection and oocyst production in feces was measured on d 5-6, 6-7, 7-8, 8-9 and 13-14.

Secondary infection. Fourteen d after the primary infection, poults from two of the cages in each treatment were either challenged with 50 x 10^3 sporulated oocysts per bird or not challenged. Thus, there were four treatments: infected challenged (IC), infected unchallenged (IU), uninfected challenged (UC), and uninfected unchallenged (UU). Weight gain was recorded from d zero through six post challenge and oocyst production measured in the feces on d 5-6, 6-7, and 7-8 post challenge.

Analysis of blood samples. Blood samples were collected from three pre-selected birds from each cage on d zero, four, seven, and 11 following the primary infection. A total of 12 samples were collected from each treatment. The blood was used for measurement of total leukocyte number using an automated hematology analyzer (Cell-Dyn 35500 SL system, Abbott, Abbot Park, IL). Blood smears were prepared, stained with Wrights stain and used for differential leukocyte count (DLC) to determine the proportion of lymphocytes, heterophils, monocytes, eosinophils, and basophils. The concentration of individual leukocyte populations was calculated using the total WBC concentration estimated by the automated hematology analysis and the percentages of individual leukocytes estimated by manual differential leukocyte counts.

Peripheral blood mononuclear cells (PBMC) were isolated from the remaining blood by density gradient centrifugation using Fico / Lite LymphoH 1.077 (Ficoll; Sigma). The cells were subjected to direct immunofluorescence staining using specific monoclonal labeled antibodies. The antibodies employed were mouse anti-chicken CD4-FITC (clone CT-4) and mouse anti-chicken CD8-PE (clone 3-298) (Southern Biotechnology Associates Inc., Birmingham, AL) known to cross-react with turkey CD4 and CD8, respectively (Li et al., 1999). The percentages of CD4+ and CD8+ live cells in the PBMC population were determined by flow cytometry using a Becton Dickinson FACSort equipped with a 488-nm argon laser (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Experiment 2

Sixty turkey poults at 20 d of age were allocated to two treatments (three cages per treatment; ten poults per cage) – infected and uninfected. Poults in infected group were given an oral dose of 12.5 x 10^3 oocysts per poult and the poults in the control group were given sham dose of water. Poults were moved to clean cages daily starting from d five after infection.

Tissue collection. Cecal tissue samples were collected from two randomly selected birds from each cage (six poults per treatment) on d 0, four, seven, nine, and 11 following primary infection. Two one cm sections were taken from the mid-part and blind-end of each cecum. They were fixed in 10% buffered formalin for histological examination or placed in cold DPBS until snap frozen in OCT (Tissue-Tek®, Sakura Finetek Inc., Torrance, CA) in liquid nitrogen for immunohistochemistry. An additional sample was taken from the middle of one cecum, collected in RNA preservation buffer (RNA*later*®, Applied Biosystems, Foster City, CA) and stored at –20°C until used for isolation of RNA and gene expression analysis.

Leukocyte infiltration. Cecum samples fixed in 10% buffered formalin were used to make histological sections and stained with hematoxylin and eosin. The extent of leukocyte infiltration in the tissue sections was subjectively assessed by examination of sections under bright field microscope. A numerical score was assigned to each section where 0, one, two, and three represented no leukocyte infiltration, mild, moderate, or severe infiltration, respectively.

Immunohistochemistry. Cecum samples snap frozen in OCT were used for immunohistochemistry. Cross-sections of six µm thickness were obtained using a cryostat at -24ºC (Leica CM3050, Leica Microsystems, Inc., Bannockburn, IL). The sections were mounted on poly-lysine coated slides, fixed in acetone, incubated with specific monoclonal antibodies [unlabeled mouse anti-chicken CD4 (clone CT-4; IgG1); unlabeled mouse anti-chicken CD8α (clone 3-298; IgG1)] and then with secondary antibodies (biotinylated horse anti-mouse IgG) with washing after each step. The sections were washed once more and then incubated for 30 minutes with avidin-biotin peroxidase complex (Vectastain Elite ABC reagent, Vector Laboratories Inc., Burlingame, CA). The stained sections were analyzed using Image-Pro Plus 6.2 (Media Cybernetics, Silver Springs, MD) connected to an Olympus B x 50 light microscope (Olympus, Center Valley, PA) at 100x magnification.

The percentage area (% area) occupied by immunostained (brown)cells (CD4+ lymphocytes and CD8+ lymphocytes) in each of the sections from the two parts of the cecum was estimated.

Isolation of RNA, reverse transcription, and real-time PCR. Total RNA was isolated from the cecum samples stored in RNAlater using an RNeasy mini kit (Qiagen Inc., Valencia, CA). An additional DNase digestion step was included to remove any contaminating DNA. The quantity of RNA was assessed by spectrophotometry (Genesys 10, Thermospectronic, Rochester, NY) measuring absorbance at 260 nm and checked for purity by measuring its OD_{260} / OD_{280} ratio. The RNA (two µg from each sample) was reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

Changes in the relative gene expression of the chemokine CXCLi2, and cytokines IL1β, IFNγ, IL13 and IL10 in the cDNA samples was assessed by real-time PCR using an Applied Biosystems 7300 sequence detection system (Applied Biosystems, Foster City, CA). Previously published primer and probe sequences specific for the chicken and turkey, or turkey only, were used (Mayne et al., 2007). Differences in the relative expression of the target genes were quantified by the delta C_T ($2^{-\Delta\Delta C_T}$) method described by Livak and Schmittgen (2001).

DATA ANALYSIS

Statistical analysis was carried out using JMP software (version 8.0.2; SAS Institute Inc., Cary, NC). All the data were expressed as mean ± SEM for each treatment.

RESULTS

Experiment 1

Primary infection. Infected poults showed a significantly lower weight gain (198 ± 5; $P < 0.001$) compared to the uninfected poults (246 ± 7). Large numbers of oocysts were produced in the feces of infected poults from d five through six followed by a decline. No oocysts were produced by uninfected controls.

Secondary infection. The poults from IC (480 ± 27), IU (535 ± 29) and UU (579 ± 7) groups showed a significantly greater ($P < 0.005$) weight gain when compared to poults from UC (307 ± 28). Large numbers of oocysts were produced in the feces of uninfected challenged poults. In contrast few oocysts were produced in infected challenged birds and none were produced in unchallenged controls. This indicates that using weight gain and oocyst production as a criterion for protection, immunity had developed to *E. adenoeides*.

Leukocyte profiles. Total WBC concentration in the peripheral blood was found to be significantly elevated on d seven and 11 in infected poults compared to controls. The concentrations of lymphocytes and monocytes were elevated on d seven and eosinophils on d 11 following primary infection in infected poults. There were no differences in heterophil and basophil concentrations between infected and uninfected poults at any of the time points examined. With the exception of increased percentages of eosinophils on d 11, infection was not associated with alterations in the proportions among WBC populations. Comparison of CD4- and CD8-defined lymphocyte subpopulations in the blood of infected versus uninfected poults revealed higher concentrations of CD4+ lymphocytes on d 11, lower concentrations of CD8+ cells on d 4, and higher concentrations of CD8+ cells on d 11 of infection, as well as elevated ratios of CD4+- to CD8+-lymphocytes in infected birds on d four and 11.

Experiment 2

Leukocyte infiltration. The cellular infiltration, consisting especially of mononuclear cells was significantly higher in infected poults on d four, seven, nine, and 11 compared to controls. The scores in infected poults were 2.7, 2.0, 1.8, and 1.7 and in uninfected poults were 0.2, 0, 0.2, and 0.3 on d four, seven, nine, and 11 respectively.

Immunohistochemistry. The percent area occupied by CD4+ lymphocytes in cecum was significantly higher on d nine, 11 in infected poults (1.21 ± 0.05 on d 9; 1.59 ± 0.06 on d 11) compared to controls (0.93 ± 0.05 on d 9; 1.05 ± 0.11 on d 11). The percent area occupied by CD8+ lymphocytes was significantly higher in infected poults (1.98 ± 0.07) compared to controls (1.55 ± 0.12) on d 11.

Real-time PCR. Mean fold changes in chemokine and cytokine expression in comparison to the calibrator are presented in Fig 1. The mean fold change of CXCLi2 in infected poults was significantly increased on d four and seven compared to uninfected poults. The expression of IL1β and IL13 was significantly elevated in infected poults on d seven, expression of IL10, and IFNγ was elevated on d four compared to uninfected poults.

CONCLUSION

Based on the weight gain and oocyst production during the two phases of Experiment 1, it was concluded that poults developed immunity to *E. adenoeides* infection. The alterations in the concentrations of WBC populations observed during primary infection with *E. adenoeides*, reflect the

mobilization and recruitment of cells in response to infection. The increase in peripheral blood lymphocytes and monocytes on d seven together with the increased CD4+- to CD8+- lymphocyte ratios on d four and 11, and the increase in CD8+ lymphocytes on d 11 collectively point towards initiation of cell-mediated immunity. Increased concentration of eosinophils on d 11 suggests recruitment and participation of this granulocyte in the effector phase of the immune response. As eosinophils are generally known to have specialized in the elimination of extracellular parasites, their recruitment in response to *Eimeria* infection is not surprising and is reflective of directed anti-parasite inflammatory immune activity (Abbas et al., 2010).

The increased infiltration of mononuclear leukocytes, greater numbers of T helper and cytotoxic lymphocytes, together with the observed cytokine expression patterns in the ceca in Experiment 2 indicate that a primary *E. adenoeides* infection in the turkey stimulates inflammatory and cell mediated immune activities at the site of infection. The cytokine expression profiles during the early phase of *Eimeria* infection may be attributed to inflammatory activities of local macrophages, injured tissue cells, mast cells, NK cells, IEL, and epithelial cells as well as recruited macrophages and lymphocytes. The arrival of CD4+ and CD8+ lymphocytes on d nine and 11, together with a variable increase in IFNγ expression, appears to mark the adaptive phase of the response to *Eimeria* infection in the cecum.

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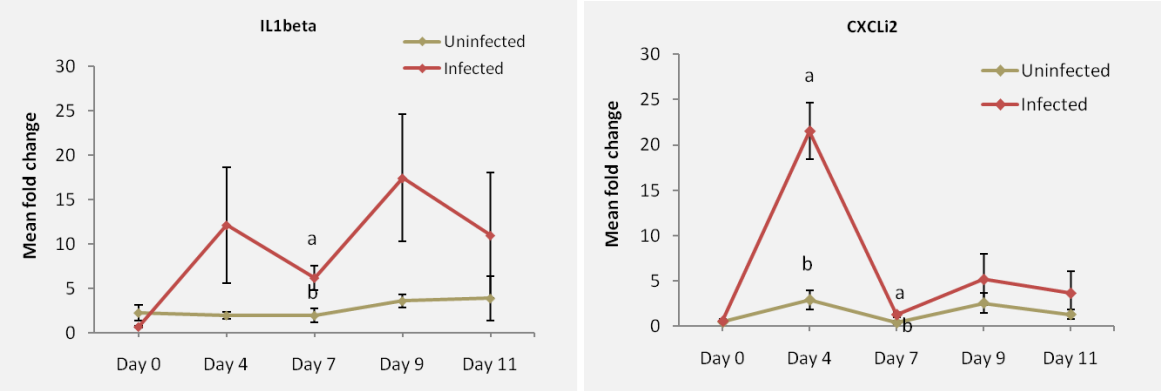
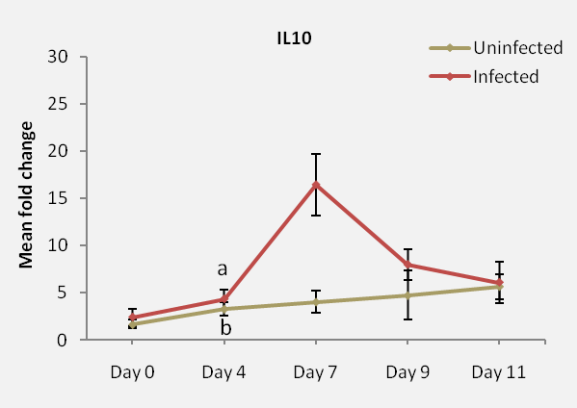
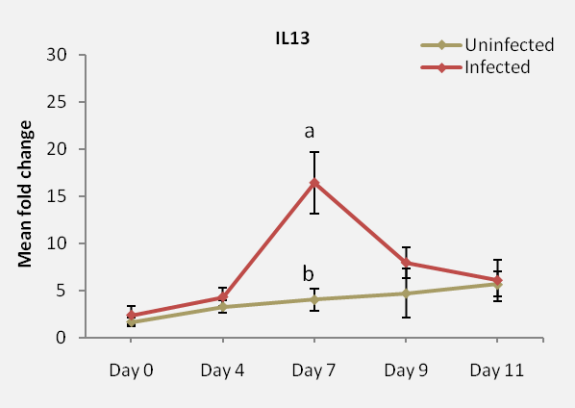
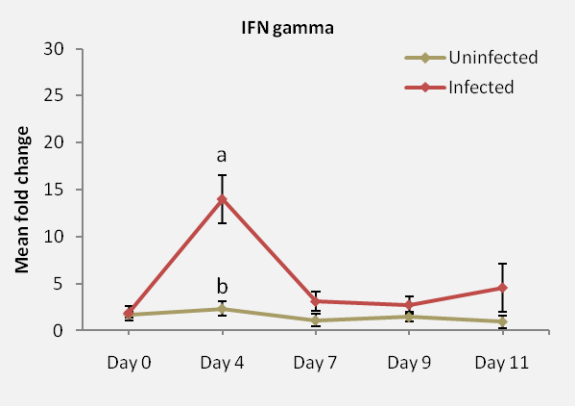
Figure 1. *E. adenoeides* compared with uninfected controls. The poults at 20 d of age were infected with 12.5 x 10^3 oocysts of *E.adenoeides* or given sham dose of water. They were moved to clean cages daily from d five of infection, to prevent re-infection with oocysts that were shed in the feces. Relative gene expression was measured by Real Time RT-PCR. The data were analyzed using the $2^{-\Delta\Delta C}{}_T$ method and expressed as fold change in comparison to the calibrator sample. For each treatment, values are the mean ± SEM for six poults.









DRUG RESPONSIVENESS OF SOME RECENT ISOLATES OF COCCIDIA FROM US BROILER FARMS

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SUMMARY

Coccidiosis control has been done by the use of in-feed pharmaceutical agents for several years. Pharmaceutical companies and broiler production companies have implemented programs to maintain or spare the efficacy of the commonly used drugs; however, sometimes judicious uses of these products are not being practiced. This exercise showed that overuse or indiscriminate usage can erode drug efficacy. Although the product may show efficacy against one isolate of the same species of coccidian, it may show reduced efficacy or complete loss of efficacy from another source.

INTRODUCTION

Coccidiosis of chickens is a disease of significant importance. The endogenous stages of the eimerian parasites may cause destruction to host tissue and substantial tissue damage may impair gastro-intestinal efficiency and even mortality. Controlling coccidiosis is achieved via the use of in-feed agents or by the use of biologics. The use of in-feed agents has been the norm for many years; however, it has become more apparent that there is erosion in the effectiveness of some of the commonly used drugs. Most of the current isolates were secured from facilities that had experienced reduced performance or complained of enteric related issues.

MATERIALS AND METHODS

Coccidia were isolated from the major broiler production areas. A determination of the predominant species was made. The isolates selected had *E. maxima* as the predominant or only species with the only exception being the second Delaware sample. The dose level for *E. maxima* isolates was 30,000 sporulated oocysts per bird. The trials terminated between 156-160 h post-inoculation (pi). Medicated feed was provided to the designated groups 36 to 48 h before inoculation and fed until termination. Birds were weighed at the day of inoculation and at termination.

All birds were euthanized, and intestines scored for gross lesions using a zero to four scale, indicative of the severity. Following the gross evaluations of the intestines, wet mount scrapings were taken and examined with a light microscope at 100x magnification. The level of parasite burden was determined using a zero to four scale.

RESULTS AND DISCUSSIONS

The current data showed that the effectiveness of the drugs might be related to the sources and/or the types of coccidiosis control programs being used. The isolate from NC was least sensitive to Monteban but most sensitive to Nicarb, whereas the isolate from SC was more sensitive to Monteban and less sensitive to Nicarb. The isolate from NC was from a program that extensively used Monteban as a part of their coccidiosis control program and the SC isolate was obtained from a program that had used Nicarb for an extended period before switching to another product. This exercise showed variability among the drug responsiveness to the different isolates of *Eimeria maxima*.

BLOOD BIOCHEMICAL CHANGES IN BIRDS WITH INCLUSION BODY HEPATITIS AND THE EFFECT OF SUPPORTIVE TREATMENTS DURING OUTBREAKS

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SUMMARY

Clinical cases of inclusion body hepatitis were investigated during the summer of 2010 using and iSTAT-1 portable biochemical analyzer. Significant changes included metabolic acidosis, hypocalcemia, and in some cases hypoglycemia. Glucose, sodium bicarbonate, and calcium were evaluated as supportive treatments. Feed analysis indicated a low electrolyte balance in the feed compatible with the metabolic acidosis. These observations may help in better understanding the pathogenicity of inclusion body hepatitis in cases where the presence of adenovirus is not sufficient per se to produce mortality.

Inclusion body hepatitis is a re-emerging disease in poultry in Canada. We experienced our first cases in 2001 after breeder companies started using autogenous vaccines and *Salmonella* control programs resulted in much cleaner grow-out barns. The end result was an increase in naïve flocks for IBH antibodies and progeny negative for IBH maternal antibodies. A second outbreak occurred in 2007 with a change in the serotype that the breeders were naïve to. During these outbreaks the disease could be followed for five to six wk after the first case from a breeder flock. A system for monitoring antibodies in breeders was used and measures to insure seropositive breeder flocks reduced the incidence of inclusion body hepatitis in the progeny. In 2010, new episodes of inclusion body hepatitis were observed in certain regions of the province of Quebec through provincial diagnostic laboratories. These cases were observed from different hatcheries and did not show the previous sequence of five to six wk of chicks with problems.

During the outbreaks, blood samples were analyzed in birds showing clinical signs and birds without clinical signs. Metabolic acidosis was a consistent finding in affected birds. Hypocalcemia and hypoglycemia were also highly prevalent. The following cases were evaluated and supportive treatments were instigated for the observed biochemical changes. A follow-up biochemical test was evaluated and zootechnical results were recorded.

Considering recent publications on the difficulty of reproducing inclusion body hepatitis with adenovirus by itself, we believe that factors favoring

metabolic acidosis, hypoglycemia or hypocalcemia can play a role in the severity of inclusion body hepatitis. The aim of this paper is to stimulate further research on the factors that may contribute to inclusion body hepatitis syndrome.

MATERIALS AND METHODS

Samples. Birds were randomly selected and identified to be with or without clinical signs of inclusion body hepatitis and venous blood was pulled from the wing vein using a one cc tuberculin syringe with a 25G x 5/8'' needle. The blood sample was immediately dispensed into CG8+ or EC8+ cartridges according to manufacturer's recommendations.

Biochemical analysis. Biochemical analysis was done on i-STAT 1 handheld Clinical analyzer (Heska Corporation, Loveland, CO, Abbott Point of care inc., East Windsor, NJ) using CG8+ or EC8+ cartridges.

Feed analysis. Feed analysis was done on 500 g of feed bins at the time of the visit. The feed sample was analyzed at the Shur-Gain laboratory, St-Hyacinthe, Qc and analyzed according to the following reference methods: sodium, potassium, calcium, phosphorous, magnesium MA#8, protein MA#2/Ma#20, chloride AOAC 969.10

Data gathering. Data for production and mortality were gathered from different logs on the farm and biochemical results were compiled using an Excel spreadsheet. The results were compared to our database for 2010 for interpretation in comparison with normal values for the type of production and the age (7 to 35 d). The data was divided into groups and the average and standard deviation for each group were calculated.

Treatment. Treatment consisted in the addition of 125 g of sodium bicarbonate plus 400 g of sugar in 576 L of drinking water.

RESULTS

Table 1 and Table 2 show the results of blood parameters and feed analysis respectively.

Results of birds with clinical signs from affected farms showed a metabolic acidosis with a respiratory compensation. The affected birds also showed a severe hypocalcemia and hypoglycemia. Treatment with sugar and sodium bicarbonate reduced the metabolic acidosis and increased ionized calcium. The treatment also reduced the previously reported ten d spike in mortality associated with inclusion body hepatitis outbreaks.

The metabolic acidosis in this study is associated with lower sodium levels in the feed and a slightly lower electrolyte balance. Birds with hypoglycemia in these inclusion body hepatitis cases were older than those reported for spiking syndrome. Calcium levels in the feed do not explain the ionized calcium levels observed in the blood.

The lower electrolyte balance observed in the feed of the birds after the water treatment can partially be explained by lower protein, soy, and potassium as birds get older. The high standard deviation observed in this data set is associated with very low sodium (0.08%), potassium (0.28%), and electrolyte balance (33 meq) levels in one case.

DISCUSSION

Without supportive treatment, hypoglycemia and hypocalcemia can rapidly kill birds. Metabolic acidosis can be compensated by respiratory pCO_2 control that may explain the observation of panting birds as a clinical sign of inclusion body hepatitis.

Clinical expression of adenovirus induced inclusion body hepatitis has been associated with immunosuppressive diseases such as Gumboro disease, chick anemia virus, or spiking syndrome. Adenoviruses are known to elicit a rapid immune response that may explain the high number of birds with antibodies and the low number of cases with clinical mortality. Hypoglycemia may further increase the pathogenicity of inclusion body hepatitis by hindering the immune system and allowing more viral replication.

Even though the electrolyte balance was marginally lower in the feed, the very low sodium levels may have hindered compensation mechanisms to excrete excess anions. The liver plays a major role through the bile cycle in the excretion and reabsorption of sodium and bicarbonates. Low intake of sodium can become critical if excretion is increased.

In this case, a source of calcium could have helped with the hypocalcemia but finding sources of calcium that do not react with sodium bicarbonate in the water needs more research because most soluble calcium sources are acid based.

CONCLUSION

A better knowledge of biochemical changes associated with inclusion body hepatitis may lead to research and development of preventative strategies. Further research in a controlled environment is needed to understand the effect of sudden changes in electrolyte and acid-base disorders on the pathogenicity of inclusion body hepatitis.

Table 1. Results of blood parameters.

Parameter	Database (7 to 35 d)	Birds from affected farms with clinical signs	Birds from affected farms without clinical signs	Birds from affected farms after treatment
N	166	47	29	16
Avg age (d)	19.68 +/-8.6	20.17 +/-5.15	19.58 +/-6.53	21.50 +/-7.35
Ht (%)	21.43 +/-3.45	18.93 +/-3.90	20.48 +/-1.97	18.63 +/-3.42
vpH	7.375 +/-0.09	7.366 +/-0.10	7.387 +/-0.07	7.355 +/-0.12
$vPCO_2$ (mmHg)	46.43 +/-9.59	34.44 +/-14.73	43.49 +/-8.87	40.43 +/-11.01
$vHCO_3^-$(mmol/L)	26.998 +/-4.12	19.219 +/-6.17	25.917 +/-6.82	22.843 +/-6.82
vBeecf (mmol/L)	1.800 +/- 4.99	-6.135 +/-6.48	0.931 +/-4.78	-2.714 +/-8.21
Na^+ (mmol/L)	144.29 +/-5.36	144.17 +/-3.93	144.90 +/-7.16	143.38 +/-3.81
K^+ (mmol/L)	5.53 +/- 0.76	5.43 +/-0.90	5.33 +/-0.59	5.58 +/-0.92
Ca^{++} (mmol/L)	1.381 +/-0.118	1.075 +/-0.271	1.433 +/-0.083	1.274 +/- 0.253
Glu (mmol/L)	14.101 +/-2.09	8.136 +/-6.34	14.213 +/-2.00	10.194 +/-5.45

Table 2. Results of feed analysis.

Parameter	Database (7 to 35 d)	Feed from affected farms with clinical signs	Feed from affected farms without clinical signs	Feed from affected farms after treatment
N	131	31	32	16
Na (%)	0.24 +/- 0.02	0.16 +/- 0.02	0.14 +/- 0.02	0.12 +/- 0.03
K (%)	0.73 +/- 0.08	0.71 +/- 0.08	0.70 +/- 0.09	0.68 +/- 0.24
Cl (%)	0.25 +/- 0.10	0.24 +/- 0.04	0.22 +/- 0.05	0.26 +/- 0.02
Ca (%)	0.94 +/-0.14	0.88 +/- 0.08	0.87 +/-0.12	0.96 +/-0.43
Total P (%)	0.66 +/-0.34	0.60+/- 0.09	0.59 +/- 0.11	0.60 +/- 0.24
Electrolyte balance (meq)	194 +/-24	184 +/-27	181+/-29	158+/-76

ANTIMICROBIAL SUSCEPTIBILITY AND DETECTION OF SOME ANTIMICROBIAL RESISTANCE GENES AMONG *NET*B POSITIVE AND *NET*B NEGATIVE *CLOSTRIDIUM PERFRINGENS* ISOLATES OF CHICKEN ORIGIN

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SUMMARY

The minimal inhibitory concentration (MIC) of six antimicrobial agents was determined for 30 *net*B positive and 30 *net*B negative *C. perfringens* field strains of chicken origin, isolated from 2005 to 2010 in Italy in 60 different farms. The presence of tetracycline resistance genes *tet*K, *tet*L, *tet*M, *tet*A(P), *tet*Q and of

macrolide-lincosamide-streptogramin B resistance genes *mef*A/E, *erm*(A), *erm*(B), *erm*(C), *erm*(Q) was investigated in all the isolates by PCR. All strains tested were susceptible to amoxicillin and tiamulin with MICs≤ 0.5 μg/mL and≤ 4 μg/mL respectively. Susceptibility to tetracycline was found in only 16.6% of tested strains and *tet*M gene was detected in two resistant strains. 81.7% of strains showed MICs values in the range of susceptibility for tylosin (0.125-0.25 μg/mL) but slightly resistant (2-16 μg/mL) and highly resistant (≥ 512 μg/mL) bacterial sub-populations were observed. The investigation of *erm*Q, *erm*B and *mef*A/E genes proved to be a reliable tool to asses *C. perfringens* resistance to tylosin and lincomycin. *Tet*A(P) and *tet*M genes were significantly related with tetracycline high MIC values. A bimodal distribution of MICs was observed for zinc-bacitracin with 80% of susceptible strains. Resistance to lincomycin was found in 71.6% of tested strains. The *C. perfringens netB* gene resulted significantly related with generally lower MIC values to amoxicillin, tylosin and lincomycin than *net*B negative strains.

INTRODUCTION

In the European Union the use of antimicrobial growth promoters (GPs) in animal feed has been banned since January 1, 2006 (1). Given the antimicrobial properties of many banned GPs, an increase of *Clostridium perfringens* associated intestinal disorders (CPAID) has been observed in poultry flocks (necrotic enteritis and disbacteriosis) (2). CPAID are controlled by the administration of antimicrobials active against gram positive bacteria such as penicillin, tetracyclines, pleuromutilins, macrolides, lincosamides and, in extra-European countries, zinc-bacitracin. The growing trend towards antibiotic use reduction in feedstuffs suggests targeting pathogens involved in the disease. For this reason the strain should be isolated, characterized for the presence of genetic virulence markers, and subjected to drug susceptibility test. The most important *C. perfringens* virulence factor implicated in necrotic enteritis of chicken seems to be the NetB pore forming toxin (3).

According to the Clinical Laboratory Standard Institute (CLSI) the calculation of minimal inhibition concentration by agar dilution method is the reference procedure to evaluate drug susceptibility of anaerobes. Unfortunately this method is time consuming and, for this reason, incompatible with the need to quickly treat diseased chicken flocks. Therefore periodic country reports on *C. perfringens* drug susceptibility are performed (4,5,6,7).

Bacterial drug susceptibility can also be evaluated indirectly by investigating drug resistance genes. The most widespread tetracycline resistance genes in Gram positive anaerobes are *tet*K, *tet*L, and *tet*M. Furthermore *tet*A(P) gene has been found alone in genus *Clostridium* (8). The macrolide-lincosamide-streptogramin B (MLS_B) resistant genes most frequently found in genus *Clostridium* are *erm*(A), *erm*(B), *erm*(C), *erm*(Q) (9). In other Gram positive bacteria, an efflux mechanism encoded by *mef*A/E gene causes macrolide resistances (10).

In the present study we investigated and compared the drug susceptibility profiles of *net*B-positive vs *net*B- negative *C. perfringens* field strains of chicken origin by means of MICs determination and resistance genes detection.

MATERIALS AND METHODS

Bacterial strains. Thirty *C. perfringens* type A *net*B-positive and 30 type A *net*B-negative field strains isolated between 2005 and 2009 were included in the study. Bacterial strains were collected during the period 2005-2009 from 53 broiler and seven layer hens from different farms.

Strain toxinotype and *net*B gene presence have been assessed by PCR protocols previously described (11,3). All strains originated from birds affected by CPAID were isolated in Perfringens Agar Base medium (Oxoid) supplemented with SFP (Shahidi-Ferguson-Perfringens Selective Supplement, Oxoid) and sheep red blood cells (5%, v/v). Strains toxinotype an *net*B gene presence were assessed by previously described PCR protocols (3,11). *C. perfringens* isolates were stored in cryogenic vials (Nalgene) at -80 °C until use.

Minimal inhibitory concentrations. MICs have been determined by agar dilution method on Brucella Agar (Becton Dickinson) supplemented with hemin (5 μg/mL), vitamin K1 (1 μg/mL) and laked sheep blood (5% v/v) according to CLSI procedures (12). The following antibiotic standard powders manufactured for analytical purposes have been tested: amoxicillin (amoxicillin powder, A8523, Sigma), tylosin (tylosin tatrate powder, T6134, Sigma), tiamulin (tiamulin fumarate powder, 46959, Sigma), tetracycline (tetracycline hydrochloride powder, T4062, Sigma), lincomycin (lincomycin hydrochloride powder, 62143, Sigma), zinc bacitracin (bacitracin zinc salt from *Bacillus licheniformis*, B5150, Sigma). Standard powders have been solubilised according to the manufacturers' instructions and serial two-fold dilutions of antimicrobial agents incorporated into Brucella Agar with antibiotic concentrations ranging from 0.016 μg/mL to 512 μg/mL.

Stored *C. perfringens* strains have been inoculated on Columbia blood agar plates (Oxoid). After overnight anaerobic incubation at 37 °C, isolates have been suspended in Brucella broth supplemented with hemin (5 μg/mL) and vitamin K1 (1 μg/mL)

(Conda) to a 0.5 McFarland standard density. Two µL of the bacterial suspensions have been used as inoculum. Two batches of MIC tests have been performed, and, in order to estimate repeatability, three reference strains (*C. perfringens* ATCC 13124, *C. difficile* ATCC 700057, *B. fragilis* ATCC 25285) have been run in each batch. The plates have been observed after 48 h of incubation at 37 °C in anaerobic conditions.

Detection of resistance genes. Five *C. perfringens* colonies of each strain have been resuspended in PBS. The Magmax Total Nucleic Acid Isolation kit (Ambion) and the Microlab Starlet automatic extractor (Hamilton) have been used for DNA extraction. *Tet*, *erm* and *mef*A/E genes have been investigated by PCR protocols previously published (8,10,13,14,15). *Tet* and *erm* genes positive controls have been kindly provided by Dr Schwaiger (Microbiology Department, Munich University), Dr Morelli (Agricolture Faculty, University of Piacenza) and Dr Del Grosso (Department of infective, parasitic and immunomediated diseases, National Institute of Health, Rome).

Data analysis. To describe the results, the 50th and 90th percentile (referred to as MIC_{50} and MIC_{90}), and the geometric mean of the MICs have been calculated. To investigate significant differences of MICs values among *net*B positive and *net*B negative *C. perfringens* strains, the Mann-Whitney U-test has been used. Fisher test has been chosen to evaluate if different MICs profiles are related to the presence/absence of the investigated resistance genes. The same statistical test has been used also to investigate possible differences of resistance genes distribution among *C. perfringens net*B positive and *net*B negative populations.

RESULTS

MICs. MICs results are displayed in Table 1. No significant differences of MIC values have been observed among *net*B-negative and *net*B-positive strains for tiamulin, zinc-bacitracin and tetracycline ($P>0.05$). Whereas, a significant difference has been assessed among the two *C. perfringens* sub-populations for amoxicillin, tylosin and lincomycin ($P<0.05$) with higher MIC values observed for *net*B negative strains than *net*B positive. In order to verify the robustness of this observation, the same statistical analysis has been repeated without MICs values considered "outliers" and the same result has been obtained.

MIC values of *C. perfringens* ATCC 13124 for tiamulin, amoxicillin, tylosin, zinc bacitracin resulted similar to MIC geometric means of field strains. On the contrary lincomycin and tetracycline MIC values were higher for field strains than reference *C. perfringens.*

Antimicrobial resistance genes. PCR results concerning the investigated resistance genes are shown in Table 2. No strain tested positive for *tet*K, *tet*Q, *erm*A and *erm*C. *Tet*A(P) is the most widespread resistance gene in the *C. perfringens* strains included in the study, in according with the common high MIC values of tetracycline observed. Tetracycline susceptible strains did not carried any of the tested *tet* genes.

Seven of ten strains showing MIC ≥ 4 µg/mL for tylosin, tested positive for at least one *erm* gene or *mef*A/E gene. Resistances to lincomycin were significantly correlated with the combination of *erm* and *mef*A/E genes ($p<0.05$).

Resistance genes distribution did not result significantly related to the presence/absence of *net*B gene.

DISCUSSION

In this study, drug resistance of two *C. perfringens* populations characterized by a different genetic virulence marker (*net*B) has been investigated by means of MICs determination for six antibiotics and by specific PCR for some tetracycline and macrolide/lincosamides antimicrobial resistance genes.

Tetracycline MIC values obtained in this study are higher than values recorded in Denmark, Norway, and Sweden, but in accordance with data from Belgium and Brazil (4,5,7). On the basis of tetracycline break points (12), 16% of strains tested susceptible, 35% tested slightly resistant and 48.8% tested highly resistant.

Detection of *tet*K, *tet*L, *tet*M and *tet*Q genes alone seems to be inadequate to asses tetracycline drug resistance of *C. perfringens* as observed by Martel *et al.* (7) for *tet*K, *tet*L and *tet*M even if the presence of *tet*M gene sporadically occurs in *C. pefringens* strains with high MIC values. On the contrary the presence of *tet*A(P) gene resulted significantly related to high MIC values ($p<0.008$). This association was even more reliable when *tet*A(P) positivity was coupled with *tet*M positivity ($p<0.004$).

Amoxicillin appears to be the most effective antimicrobial drug *in vitro* with MIC≤ 0.125 µg/mL and MIC_{90} = 0.032 µg/mL. Those values are lower than break points of ampicillin fixed in 0.5 µg/mL (12).

Tiamulin is unemployed in broiler chicken therapy because of the occurrence of toxic effects when coupled with some common anticoccidials, but it could be used in flocks vaccinated against coccidia or in layer hens farms suffering from sporadic necrotic enteritis outbreaks (16). Official break points for tiamulin are not available but strains with MIC values lower than 4 µg/mL are conventionally considered susceptible (17).

On the basis of this definition, all tested strains resulted susceptible to tiamulin.

Tylosin is widely used in poultry therapy because of its effectiveness against Gram positive bacteria and *Mycoplasma* spp. MICs values distribution showed the existence of three sub-populations clearly separated by two antimicrobial dilutions. The first population includes 81.7% of tested strains with MIC ≤ 0.25 μg/mL and it can be defined susceptible. The other two populations include 15% and 3.3% of strains with MIC values ranging from two to 16 μg/mL (slightly resistant) and ≥ 512 μg/mL (highly resistant) respectively (4).

The investigation of *erm*Q, *erm*B and *mef*A/E genes proved to be a reliable tool to assess *C. perfringens* resistance to tylosin.

71.6% of tested strains showed MIC values of lincomycin ≥ 1 μg/mL, regarded as the susceptible limit for this molecule (7). This result is in agreement with those obtained in Brazil, whereas in Belgium a lower percentage (43%) of strains tested resistant (7). In a USA country report, 100% of tested *C. perfringens* of chicken origin resulted resistant to lincomycin with MICs values higher than 8 μg/mL (6). MIC results of lincomycin were independent from the presence or absence of e*rm*B gene ($p > 0.05$), while they were dependent from *Mef* A/E, *erm*Q and the combination of the group of *erm* and *mef*A/E genes ($p < 0.05$). In particular, the presence of these last genes seemed to determine the highest MIC values.

Zinc bacitracin is not registered for use in poultry flocks in Italy but it is widely used in other non-European countries to control necrotic enteritis of chicken. A classic bimodal MICs distribution has been observed for this molecule and 20% of strains resulted resistant with MICs ≥ 16 μg/mL (18).

This study is the first report on drug susceptibility of *C. perfringens* strains isolated from chickens affected by CPAID in Italy. Periodical reports are necessary to monitor antimicrobial resistances and to promote a more responsible use of antimicrobials in poultry.

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Table 1. Distribution of minimal inhibitory concentrations, MIC50 and MIC90 of six antimicrobial agents for 60 *C. perfringens* field strains.

	MIC (μg/mL)																**MIC50**	**MIC90**
	0,016	**0,032**	**0,063**	**0,125**	**0,25**	**0,5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**		
Tiamulin					16	7	10	21	6								1	2
Amoxicillin	41	17	1	1													0,016	0,032
Tylosin				3	46			1	2	5	1					2	0,25	8
Zn-Bacitracin						2	13	31	2				5	7			2	128
Lincomycin				14	2		1	9	13	6	7			4	4		4	128
Tetracycline				2	2				6	21	21	8					8	32

Table 2. Distribution of drug resistant genes among *net*B-positive and *net*B-negative *C. perfringens.*

	***tet*K**	***tet*L**	***tet*M**	***tet*A(P)**	***tet*Q**	***erm*A**	***erm*B**	***erm*C**	***erm*Q**	***mef*A/E**
*net*B positive	0	0	0	29	0	0	0	0	3	3
*net*B negative	0	1	2	28	0	0	2	0	5	7

THE NPIP SE CLEAN PROGRAM AND THE FDA EGG RULE

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In 1989, the National Poultry Improvement Plan (NPIP) established the US Sanitation Monitored Program for egg type breeders which would eventually become the US *Salmonella* Enteritidis (SE) Clean Program. This program has been effective in controlling SE infections in the egg type breeder industry supplying chicks to the commercial layer industry.

In July 2010, the FDA Egg Safety Rule was established in an attempt to control SE infections in commercial layers. The FDA Egg Safety Rule affects 99% of US egg production and requires the facility to register and maintain required records, implement biosecurity and pest control programs, conduct environmental testing for SE at 14-16 wks and 40-45 wks, and requires mandatory diversion and egg cultures in the event of an environmental positive SE test. For the environmental testing, FDA has recognized the NPIP provision from 9 CFR 147.12 "Procedures for collection, isolation, and identification of *Salmonella* from house environmental samples, cloacal swabs and hatchery samples" as equivalent to FDA methods in accuracy, precision and sensitivity in detecting SE infections. This revised provision gained interim approval by the General Conference Committee (GCC) of the NPIP in Dec. 2010 and is currently awaiting publication as a program standard as referenced in the 9 CFR Part 147.

Another requirement of the FDA Egg Safety rule is that commercial layer pullets be sourced from breeder flocks certified under the US *Salmonella* Enteritidis Clean program of the NPIP. This program requires that breeder flocks be tested for SE through both environmental and serological methods and any positive environmental samples must be followed by bird culture for flock status determination.

The willingness of the FDA and the USDA to work together on the issue of SE control in table eggs further supports the "One-Health" concept for food safety. It is encouraging that positive interaction of government agencies could be a method of making our food supply even safer in the future.

A PRELIMINARY RETROSPECTIVE COMPARISON OF THE TRENDS AND RESULTS OF THE FDA EGG SAFETY RULE AND THE CALIFORNIA EGG QUALITY ASSURANCE PROGRAM IN REGARDS TO SE MONITORING

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SUMMARY

A retrospective study of *Salmonella* Enteritidis (SE) environmental sampling as reported by the California Animal Health and Food Safety Laboratory System (CAHFS) is reported for California egg producers from 2007 to 2011. Between July 2007 and November 2011, more than 2500 environmental drag swab accessions were collected from California layer flocks on behalf of the California Egg Quality Assurance Plan (CEQAP) and/or the FDA's egg safety rule (21 CFR 118). This study retrospectively compares trends in sampling, results of sampling, and comparisons between sampling conducted under the CEQAP and the FDA egg safety rule (FDA).

Pearson's Chi-square tests showed significant differences between the old CEQAP sampling procedure (i.e., SE sampling of chick papers and/or push-out) and the FDA sampling protocol (i.e., pre-production, mid-production, and post-molt) between the years 2007-2011. Specifically, looking at data from 2007-2011, the FDA-mandated samples were three times more likely to be positive than samples taken under the old-CEQAP program of a single sample in the life of the flock. However, for those same years, FDA mandated samples showed no significant difference in probability of being positive as samples taken according to the new CEQAP program that requires SE environmental swabs from five stages in the flock's life-cycle. Analyses of the prevalence of SE at different stages in the flock's lifecycle (chick-papers, pre-production, production, post-molt and pre-market samples) found the incidence of positive submissions to be found in the order of premarket > production > postmolt > chickpapers > preproduction. Pooled samples versus individual swabs showed no significant difference at the .05 level of confidence. Participation in the CEQAP program showed a significantly lower probability of positive results than submissions from non CEQAP participants.

Overall sampling has increased since the implementation of the FDA mandatory program, which has resulted in a higher probability that the table egg supply is safer, because the majority of flocks found to be positive had been depopulated, and the eggs were not marketed after the results were reported. However, those eggs were marketed in the time prior to the company receiving the positive result, which implies since the last negative test. The average interval between testing is 25 wk, meaning that SE positive eggs could still enter the table egg chain for some time before the flock's environment is found to be positive.

By all indications, CEQAP and EQAPs in general have been successful in SE mitigation (1). The overall prevalence of SE found in the environment of California poultry farms between July of 2007 and November of 2011 of 5.1% was consistent with previous statewide studies which showed the prevalence of SE at 10% and 2.6% in table egg flocks in California respectively (2). While it is well

documented that molting can have a significant positive effect on transmission of SE to uninfected hens (3), our results show that the post-molt samples had a lower prevalence than samples taken during production. Admittedly, the results may be skewed due to the quality of the data as reported. Because the majority of the hen's life is spent in the production phase, in all probability the results reflect reality. Hopefully, this implies that SE positive flocks were detected prior to molt and corrective measures were taken. Manure swabs are considered the best indicator of the presence of SE in the layer environment and therefore supposedly in the hens themselves, but the probability of collecting SE on the swab is not high (5,6,7). With the current CEQAP and FDA programs, an average of 25 wks\ (nearly six months) between sampling exists, which means that with the possibility of having false negative results on any given swabbing, contaminated eggs could be marketed undetected (8). The odds of detecting SE under the CEQAP program which samples at five times in the lifetime of laying hens is higher than the FDA program which samples at three times. This implies that the more frequently the flock is sampled the more frequently SE will be found.

(A more detailed analysis is being planned for submission to *Avian Diseases* as the data is refined.)

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MORTALITY IN CAGE LAYERS ASSOCIATED WITH A SPECIFIC CAGE DESIGN

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SUMMARY

Investigation of unexpected mortality in caged, layer chickens led to the discovery of a consistent traumatic injury to the heads of affected hens. In the morning, dead birds would often be found in cages vertically aligned amongst the four tiers with only one affected bird per cage. Post-mortem examination found linear skin lacerations and associated fractures in the dorsal cranium under the dorsal lobe of the comb of all birds examined, and associated five-ten mm deep trauma in the underlying brain tissue. Decapitation was extremely rare and four previous batches of hens in these cages had not had a significant problem. Post-mortem multidetector CT scanning of two affected animals demonstrated similar obliquely orientated, linear, depressed fractures of the skulls consistent with a single, severe impact force to the head. Both skull

fractures had a pattern of rounded, anterior expansion measuring approximately three mm in width. On inspection of the cages during a farm visit, this CT pattern corresponded with the size and shape of sheet metal lugs holding the feed troughs onto the cages (on which blood stains were subsequently observed). A hopper moved in front of the cages distributing feed four times per day. Based on this analysis and hypothesizing that hunger was a triggering factor, a recommendation was made to reverse the shed "lights on" and feed hopper operation times (to operate the hopper in the dark period) with instant reduction in mortality. The CT visualization allowed better understanding and communication of the nature of the injury between professionals and with the farmer, and aided the identification of the lug as the origin of the fracture. This case highlights the value of CT imaging in mortality investigation where trauma is postulated.

(The full-length article has been submitted to *Avian Pathology*.)

COMPARISON OF IMMUNOLOGICAL AND HEALTH PARAMETERS OF DIFFERENT LAYER HYBRIDS HOUSED IN AVIARY AND ENRICHED COLONY SYSTEMS

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SUMMARY

Changes in German and EU legislation have led to increased requirements for layer housing. There is a need to evaluate different housing systems, such as aviary and enriched colony systems, regarding their influence on the health performance and possible effects on the immune responsiveness of the birds. Preliminary investigations indicated that the genetic background of the layer hybrid may also have a significant influence on these parameters in correlation to the different housing systems. In this study we monitored different immunological and health parameters during three consecutive placements of Lohmann Brown (LB) and Lohmann Selected Leghorn (LSL) layer chickens. Birds were housed on the same farm under the same management conditions in an aviary or enriched colony system. Differences were seen in the cannibalism rate not only between housing systems but also between hybrids. On the other hand, the data indicated that the differences between the evaluated immunological parameters were mainly due to the genetic background.

INTRODUCTION

Due to EU legislation, (1999/74/EC) conventional cages have been banned, and alternative housing systems for laying hens will be required in European countries in 2012. This directive had already been implemented in national law by the German government in 2006 leading to the complete ban of conventional cages since the beginning of 2010. These changes led to the development of enriched colony systems (ECS) for layers, which meet the increased requirements for housing layers in Germany. Besides the required space of 890 cm^2/hen, small colony systems are enriched with nest boxes, perches at two different levels, and a dust bath area (Tierschutz-Nutztierhaltungsverordnung, 2006; 1). The alternative housing systems allow the hens to perform their natural behavior, but possibly the hygienic status may be more difficult to control (2). Accordingly, more studies are needed to compare different alternative housing systems with regard to their influence to the animal's immunological status and responsiveness, which may significantly be affected by stress (3), or additional infectious pressure. Genetic background of the animals may play a role in the immunological responsiveness of hens (4, 5). The goal of this study was to investigate the influence of different housing systems such as ECS

and aviary system (AS) on the health and immunological status of LB and LSL laying hens kept under the same management conditions on one farm during three consecutive placements.

MATERIALS AND METHODS

Housing systems and animal trials. All animal trials were approved by the animal welfare committee of Lower Saxony. A total of three trials were conducted over a laying period of about one year each between 2008 and 2011. For these trials, young LSL and LB hens between 16 and 18 wk of age were obtained from a commercial source. They had been reared on the floor on deep litter (trial 1, 2) or cages (trial 3), and were exposed to a standard vaccination procedure. *Ad libitum* feeding was provided and water was supplied *ad libitum* per nipple drinkers in each trial. In each trial a total of 684 LB and 666 LSL hens were distributed in enriched colony systems (Big Dutchman, Germany standard; ECS) in groups of 36 or 54 animals each with 890 cm^2 of space available per bird. The different genetics and group sizes were evenly distributed throughout the barn within three tiers. 2510 LB (trial 1) and about 1800 LB hens (trials 2, 3) were housed in the Natura and Natura 60 aviary system (AS) (Big Dutchman, Vechta, Germany), respectively. Birds were routinely vaccinated against Newcastle disease and infectious bronchitis during the trials. Serum and blood samples were collected at about 24, 48, and 70 wk and investigated for serum antibodies and different immune cell populations such as heterophils, lymphocytes, monocytes, and thrombocytes as well as the heterophil to lymphocyte ratio. During the trials, moribund or dead chickens were necropsied and pathological lesions determined.

Serological response. Serum samples were collected from 30 animals per genetic background and housing system. They were investigated for antibodies against *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, infectious bronchitis virus, avian encephalomyelitis virus, and avian Metapneumovirus (aMPV) using commercial ELISA systems from Synbiotics (Pfizer, France) and LDL (Leipzig, Germany).

Phenotyping and estimation of the heterophil/lymphocyte ratio. Peripheral blood leukocytes were collected (n = 10/genetic background and housing system) and analysed by flow cytometric analysis as previously described (6).

RESULTS AND DISCUSSION

All groups of layers in the different housing systems showed expected laying performance of above 84 % determined from the beginning of lay. The health and immunological parameters were compared between LSL and LB chickens housed in the ECS, and between LB chickens housed in the ECS and AS using the Statistics 9 software (USA). Significant differences were seen in the cannibalism rates between LB and LSL hens in the ECS during the first two trials allowing the speculation of genetically based differences ($P < 0.03$) between animals dealing with the housing conditions. In the third trial, the cannibalism rate was very low in both LB and LSL chickens (n =7/group), possibly due to changes in light program, rearing conditions, and changes in mite prophylaxis strategy implemented in this trial compared to the first two trials.

In trial one, LB hens housed in AS showed significantly lower cannibalism-based mortality compared to the LB hens housed in the ECS, especially during first two third of the laying period ($P < 0.05$). The older model of the AS was replaced by the newer AS, which resulted in increased cannibalism rates in AS-housed LB chickens in trial 2 and 3. This was possibly due to insufficient experience of the animal care takers with this new system because the older AS have had also low cannibalism-based mortality rates in previous layer flocks on the same farm before the beginning of the presented experiment.

Other major necropsy findings besides mortality due to cannibalism were traumata, cachexia, nephrosis, and inflammatory lesions of the oviduct and ovary. These lesions contributed with 0.2 to 2.3 % to the total mortality in trial 1 and 2. In trial 3 we also found ulcerative gastritis in the LB hens of both housing systems in about 1 % of necropsied birds, which allows the speculation of additional stress factors especially during the early time of lay.

The humoral immune response was detected against a selected group of pathogens to determine possible causes of depression of laying performance, increased mortality, and to determine if the antibody response may vary depending on the housing system and genetic background as it was suggested by previous studies (4). All birds had been vaccinated with the same vaccines and schedule during the rearing period, and against Newcastle disease and infectious bronchitis virus infection during their laying period. The evaluation of the ELISA results provides circumstantial evidence that birds were exposed to an aMPV- field challenge in the first trial without additional effects on the laying performance. During the consecutive trials, birds were then vaccinated against aMPV. In all trials significant differences in antibody levels against some of the selected pathogens were detected between genetic backgrounds ($P < 0.05$). These differences varied between time points of sampling within one trial. Differences in antibody levels were also occasionally seen between LB hens

housed in the ECS and AS, which varied between trials. More research is needed to confirm these observations and identify possible causes for variation.

The phenotypic analysis of blood samples indicated that the absolute number of thrombocytes remained mainly constant between groups beside some differences between LSL and LB birds at the age of 24 wk ($P < 0.05$). The numbers of monocytes were stable and comparable between different groups. Clear differences were seen in the heterophil/lymphocyte (H/L) ratios between LSL and LB birds. In all trials we saw at various time points significantly lower H/L ratio in the LSL-group compared to the LB group ($P < 0.05$). There was no clear correlation between H/L ratios and mortality rates, nor housing system within the LB genetic background as also demonstrated previously (7). H/L ratios of 0.8 and higher are indicators of high degrees of stress (8) which were detected in trial 1 and 3 in the group of LB hens housed in the ECS. More studies under field conditions are needed to bring together the effect of housing systems, genetic background of layers, and additional stress factors to really demonstrate possible synergistic or additive effects of these different factors on immunological parameters.

(The full-length article will be published elsewhere.)

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TROUBLESHOOTING HATCHERY CONTAMINATION ISSUES THAT IMPACT CHICK QUALITY - COMMON SOURCES

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INTRODUCTION

The use of 7-day mortality to assess chick quality is a common practice within the poultry industry. Typically, when the 7-day mortality for a company (or a complex within a company) exceeds 1.25% or higher for several wk, the company may seek the assistance from a veterinarian or a hatchery consultant. A close evaluation of the 7-day mortality will typically give the investigator some clues as to where to start the investigation. For example, if the first two days are significantly elevated, there may be producer culling, trauma, or a bacterial contamination issues. However, if days one through three are normal, and days four through seven are significantly elevated, then there may be water issues on the farm.

This paper will focus on a method to help troubleshoot high mortality that occurs within the first two days. Most of the time this will be a bacterial contamination issue; however, we have to first rule out

a few other possibilities. The first being excess culling of small birds. This is often seen in companies or complexes that have recently experienced severe flock uniformity issues due to RSS (Runting-Stunting Syndrome) or a feed ingredient issue. In these complexes, the producers tend to excessively cull the small (normal) birds in hopes to improve flock uniformity. The second common cause that needs to be ruled out is trauma. Most of the time, the trauma is very obvious but occasionally this may not be the case. An example of this was a case where chicks were being injured on the separator at the hatchery resulting in a small tear in the liver and a slow bleed-out. The chicks left the hatchery looking normal but died soon after reaching the farm. On post mortem examination of the dead, abdominal blood clots were noted. This high day-one mortality was a trauma issue and not a contamination issue.

Twenty-four hour mortality evaluation – a method for evaluating bacterial contamination. Once trauma and culling have been ruled out, the following procedure can help determine if a significant bacterial contamination issue exists. It can also help determine the type of bacterial challenge that may have built up within the hatchery or within the egg. Once the bacteria have been identified, the investigator can focus his investigation on areas where that particular bacterium is most commonly found.

1. During the hatch day, set aside at least 20 boxes of chicks (2000 chicks). If there is concern about a particular flock, make sure to hold some chicks from that flock. Otherwise just select a variety of flock ages, incorporating young, prime, and old breeder flocks and label the chick boxes accordingly. As a box is selected, scan through it and remove any obvious culls (large navel tags, significant trauma. . .). Only "normal" chicks should be held for the evaluation.
2. Check the boxes throughout the day for any mortality. During the night, have the night attendant check the boxes every two to three hours. If any dead are found, remove them from the box and place them in a Ziploc bag in the refrigerator, not the freezer. Label the bags according to which box the chick was removed from. A spreadsheet can be used to track the number of chicks removed from each box during each evaluation.
3. The following morning, the dead chicks can be taken (on ice) to a local lab for culture and sensitivity testing. It is always a good idea to give the lab advanced notice so they will be prepared. Request the following from the lab:
    - Take cultures from the yolk sac, liver, lung, and brain of each chick submitted. The culture should not be called negative until it has completed 48 h of incubation (some bacteria are slower to grow). We are specifically looking for *Escherichia coli* (*E. coli*), *Proteus*, *Enterococcus*, *Pseudomonas*, *Klebsiella*, and *Aspergillus*. However, any bacterial growth should be noted.
    - Request the level of contamination from each organ. For example:
        - liver: +3 Ecoli
        - brain: +1 Ecoli
        - yolk: +4 Pseudomonas
        - Lung: No growth
        - or they could use mild, moderate or severe
    - Also request an antibiotic sensitivity test on the most prevalent bacteria.

This procedure can be done on multiple days to confirm that the results are repeatable.

Interpretation of results. Below are some key assessments from this evaluation:

- Keep in mind that the boxes were scanned and all obvious culls were removed before the trial began, therefore, even one dead chick per box is significant. Since each box contains 100 chicks, one chick would equate to one percent day-one mortality.
- Is there one consistent bacterium found in multiple flocks? This would suggest a common source and would point efforts in the direction of the hatchery versus individual breeder farms. On the other hand, if we have one flock that stands out from the others, it is most likely a breeder farm issue.
- Once the bacterium is identified, we can then point the investigation in the direction of where that bacterium is most likely found. For example, if *Pseudomonas* is consistently isolated from multiple flocks, then the investigation would be directed toward water sources (water systems, hatchery water pipes, vaccination water sources, fogger nozzles…). If *Proteus* and *E. coli* are consistently isolated from multiple flocks then the investigation would be directed toward areas where eggs might get wet (overhead pipes or roof leaking on eggs, cleaning crews accidently wetting eggs, egg sweating due to temperature abuse, etc.). If *Enterococcus* is consistently found, then the investigation would be directed toward the tray wash (inadequate temperatures, inadequate cleaning of trays due to plugged nozzles, inadequate post wash disinfection etc.).

- Finally, the sensitivity test results can be used to determine if it may be possible to change or increase the dose of an antibiotic as a temporarily fix while corrective actions are being done.

CONCLUSION

This 24 h mortality evaluation was developed to confirm the presence of a significant bacterial contamination issue and, with the correct identification of the agent, provide a good direction for the investigator to focus the investigation. During this presentation, common sources for bacterial contamination will be discussed.

THE INFLUENCE OF LIGHT INTENSITY ON COCCIDIOSIS VACCINE UPTAKE IN THE HATCHERY

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SUMMARY

Successful mass application is critical to the success of any broiler coccidiosis vaccination program. All live coccidiosis vaccines require oral ingestion. A previous study done at the University of Delaware's Lasher Lab showed that the spray cabinet efficacy is approximately 88% based on oocyst shed rates five to eight d post hatch. The question remains, can we improve beyond this 88%? Using the following paper as a starting point (1), research was implemented to determine if increasing relative photo intensity at the time of spray application can have a positive impact on vaccine uptake. Table 1 is a chart of the results from the initial study.

Based on the findings in Table 1, further research has been done to determine the optimal program needed to successfully implement a light program in a commercial hatchery. The findings of these studies will be discussed in depth.

It was also determined that enhanced light intensity has a positive impact on the shed pattern of oocyst, which in turn had a direct positive impact on necrotic enteritis.

Table 1. Chick room light intensity.

Group	Light Intensity	% Change of Oocyst Shed
High	88.0 foot candle	58 % greater shed than control
Medium	21.0 foot candle	Control
Low	0.6 foot candle	52% *less* shed than control

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CURRENT ADVANCES IN IMMUNIZATION OF POULTRY AGAINST FOODBORNE PATHOGENS

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Control of foodborne pathogens remains a major challenge for the poultry industry and of critical importance to public health. Efforts to minimize or eliminate food-borne pathogens, such as *Salmonella*, *Campylobacter,* and *Listeria*, must include preharvest and postharvest strategies. Vaccines developed for poultry have shown to be effective in reducing *Salmonella* in the environment and in reducing vertical transmission of infection. *Salmonella* vaccination programs, along with other control measures, have been implemented successfully in providing a last line of defense for the bird against infection by field strains. To date, no other vaccines are commercially available for poultry for the prevention of human bacterial enteropathogens such as *Campylobacter* and *Listeria*.

Salmonella sp. and *Campylobacter jejuni* cause the largest number of foodborne illness in the US, with poultry and poultry products attributed as the major source of infection. Although *Campylobacter* infections have decreased 27% since 1996-1998, the number of incidences that occurred in 2010 ranks second to *Salmonella* cases at 13.6 per 100,000 (3). *Salmonella* infection was the most common infection reported (17.6 illnesses per 100,000) and was associated with the largest number of hospitalizations and deaths. The incidence of listeriosis, caused by *Listeria monocytogenes*, has also decreased 38% since 1996-1998 (3). Foods associated with transmission of *Listeria* to humans become contaminated during processing. Although *Listeria* contamination is only infrequently acquired on the farm and carried into the processing plant, equipment and the plant environment become contaminated and serve as reservoirs to cross contaminate carcasses (4, 6).

The USDA FSIS recommends the use of vaccination programs for poultry as one of several interventions to reduce foodborne pathogens (10). Live and inactivated vaccines to reduce colonization of the gut and reproductive organs by *Salmonella* are commercially available and have been demonstrated both by controlled and field studies to effectively reduce vertical transfer of *Salmonella* carriage and to confer protective immunity to progeny of vaccinated breeders through IgY maternal antibody (8, 15, 27). Studies in hens undergoing molt have shown significant protection against challenge with wild-type *S*. Enteritidis after one spray application of a live *Salmonella* vaccine prior to feed withdrawal (16). Hassan and Curtiss (14) demonstrated that vaccination of chickens with a live *S*. Typhimurium vaccine provided significant cross-protection to challenge with several serotypes in the B, D and E *Salmonella* serogroups.

A recent study determined the effectiveness of a vaccination program combining a live *Salmonella* vaccine with an inactivated *S*. Enteritidis vaccine. Two groups of birds were either vaccinated with AviPro® SE-109 SE-4 (Killed Vaccine: KV) at 12 and 16 wk of age, or vaccinated (by coarse spray) with AviPro Megan®Egg at nine wk of age followed by AviPro SE-109 SE-4 at 12 and 16 wk of age. Similar groups of birds were held as nonvaccinated controls. After independent groups of vaccinated and nonvaccinated birds were orally challenged with three different wild type *Salmonella* strains at 21 wk of age, internal organs and ceca were obtained a wk later and cultured for the respective wild-type *Salmonella* challenge organisms. The level of protection was assessed by determining the difference in the percentage of birds in each vaccination program whose organs were completely cleared of the wild type challenge strain compared to nonvaccinated birds. Figure 1 shows the synergistic effect of priming young birds with the live *Salmonella* vaccine to enhance protection against challenge with wild type *S*. Typhimurium, *S*. Heidelberg, and *S*. Enteritidis when groups of birds given only the inactivated vaccine were compared to groups of birds given a combination of the live *Salmonella* vaccine and the inactivated vaccine.

Campylobacter remains a challenge for the broiler industry. Risk factors for the occurrence of *Campylobacter* were identified in broiler flocks as age and flock size (17), animals in the vicinity of the broiler house, livestock other than chickens on the farm, a down period of less than 14 d, dividing the flocks for slaughter (13), and the practice of batch depletion (12). Stern *et al*. (23) reported enhanced on-farm biosecurity practices and freezing of carcasses from *Campylobacter* positive flocks contributed to the significant drop in poultry associated campylobacteriosis in Iceland. Government regulations and implementation of stringent biosecurity practices in primary production were effective in preventing *Campylobacter* infection of flocks and was directly correlated to a 74% reduction in

campylobacteriosis attributed to poultry in New Zealand (21). Several experimental approaches, like the reduction of colonization by competitive exclusion, bacteriocins, and application of bacteriophage are currently under investigation for their effectiveness in reducing *Campylobacter* in primary production (11, 22, 24). Although killed whole-cell *Campylobacter* vaccines have had limited success (7), Wyszynska *et al*. (26) reported more than a six log reduction of *Campylobacter jejuni* after challenge of birds vaccinated with a live recombinant Δ*cya* Δ*crp* *Salmonella* vaccine strain synthesizing the highly conserved immunodominant CjaA protein. Buckley and coworkers (2) found that an attenuated Δ*aroA* *Salmonella* Typhimurium strain synthesizing CjaA fused to TetC reduced the challenge *C. jejuni* organisms in the ceca by 1.4 log in chickens. More recently, Layton *et al*. (19) found that oral vaccination with Δ*aroA htrA* S. Enteritidis synthesizing CjaD, an outer membrane protein, reduced *C. jejuni* colonization in the ileal mucosal samples by 4.8 log to undetectable levels (19).

The Centers for Disease Control demonstrated a direct epidemiological link between human listeriosis and the consumption of undercooked poultry (20). *Listeria* is widely distributed in nature (25) and it would be expected that poultry could be exposed during live production. However, the development of poultry vaccines against *Listeria monocytogenes* would not be economical due to the infrequent and low isolation of the organism from live birds (1, 9). The antimicrobial and protective mechanism of probiotic bacteria against *Listeria* has been investigated. Corr *et al*. (5) demonstrated that bacteriocin-secreting *Lactobacillus salivarius* produced a consistent reduction of one to two logs in internal organs of mice challenged with over 10^9 CFU of *L. monocytogenes.* More interesting was evidence that supported direct antagonism as the mode of action involved in protection against *L. monocytogenes* as early as 30 minutes after administration of the *L. salivarius*. Recent studies by Koo and coworkers (18) showed that *Listeria* adhesion protein (LAP) synthesized by recombinant probiotic *Lactobacillus paracasei* was able to interact and attach to heat shock protein on the surface of intestinal cells just as *Listeria* would. The *L. paracasei* physically crowded out *Listeria* organisms and decreased the number of *Listeria* cells that passed through intestinal cells by 46%. The direct human use of bacteriophage was approved in 2006 by FDA when a phage preparation targeted against *L. monocytogenes* was allowed for spraying meat thus opening the way for other phages to be recognized as having GRAS (generally recognized as safe) status (ListShield, http://www. Intralytix.com).

Extensive research and field trials have identified a wide range of management and intervention measures for the reduction of human enteropathogens from poultry. Vaccination of poultry has been shown to reduce carriage of *Salmonella* into the processing plant. While many strategies for reducing foodborne pathogens have been proven efficacious in laboratory research, the conduct of true field trials under commercial settings requires continued research.

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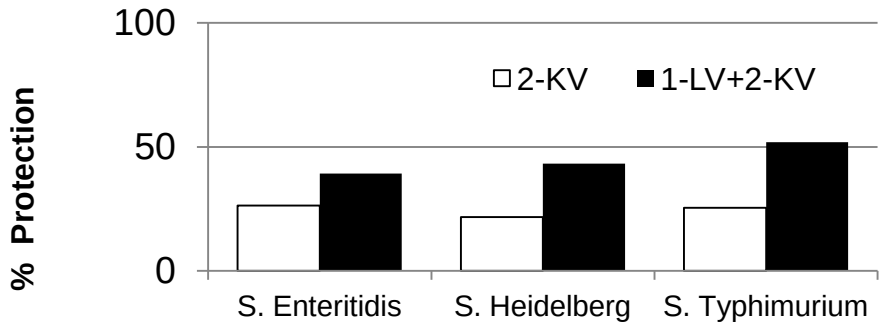
Figure 1. Protection (%) of birds provided either inactivated SE vaccine alone or in combination with a live *S.* Typhimurium vaccine.



SALMONELLOSIS IN BROILER CHICKENS: AN INTEGRATED RESPONSE

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SUMMARY

High mortalities, an increased number of daily culls and mobility, uniformity, and performance issues were seen in commercial broiler chickens predominantly associated with *Salmonella enterica* serovar Enteritidis and *E. coli*. Environmental testing was done to estimate the prevalence of *Salmonella* Enteritidis (SE) among local broiler breeder flocks. A significant number of flocks were found positive for various serotypes of Salmonellae. Canadian Poultry Consultants (CPC) developed a Salmonella Prevention and Control Program for its clientele, to be activated particularly in SE positive flocks. The CPC program was introduced to Hatching Egg Producers through local group meetings. This program has been applied to certain SE positive flocks.

INTRODUCTION

Salmonellosis is an important disease of poultry that can also affect the health of humans. In 2008, the British Columbia Center for Disease Control reported a high incidence of SE infection in humans. Concurrently, a significant rise in the frequency of SE isolated from hatchery fluff samples was observed. It is known that some paratyphoid (PT) serotypes of SE can be deposited in the contents of eggs before oviposition (2), resulting in vertical transmission of the pathogen.

Salmonella shedding by broiler breeder chickens could be effectively reduced by systematic application of the CPC Salmonella Control and Prevention Program.

MATERIALS AND METHODS

The CPC Salmonella Control and Prevention Program was compiled and made available in printed and online form for broiler hatching egg producers. Producer meetings were organized and *Salmonella* monitoring, prevention, and intervention strategies were discussed. The CPC program included enhanced biosecurity protocols, administration of live *Salmonella* vaccine and autogenous bacterin, addition of probiotics to the feed, and acidification of the water and litter. Salmonellae serovars used in an autogenous bacterin were isolated from local broiler breeder and broiler chicken flocks. The autogenous bacterin was prepared by a commercial vaccine manufacturing company in USA.

RESULTS AND DISCUSSION

During the course of this SE event, it was observed that infection with SE had little effect on mortalities, fertility, and hatchability in adult broiler breeder chickens. Broiler hatching egg producer meetings were organized to raise awareness about this issue and to introduce the concepts of control measures. These meetings helped producers to recognize the importance of SE as a problem and

encouraged a willingness to implement intervention strategies.

In a pullet flock that was positive for SE at 14 wk, intervention strategies were applied. The same flock was retested twice prior to egg production and found negative for SE.

At two facilities, after the termination of SE positive flocks, special attention was paid to rodent control, darkling beetle control, cleaning, and disinfection before re-population. The barns were tested prior to placement and found to be negative for SE.

SE is difficult to control, particularly in multiage flocks. Rodents and darkling beetles can serve as reservoirs or mechanically maintain the organism to be carried over to the next flocks. SE can be carried by people and possibly by air from an infected existing flock to a newly introduced susceptible flock. Previous studies have shown that the environmental persistence of PT salmonellae creates continuous opportunities for horizontal transmission of infection within and between flocks. SE has been observed to survive in litter and feed for 26 months after removal of an infected flock (1). It is also likely that dust from an infected flock could remain SE positive until the infected flock is terminated and the barn is properly cleaned and disinfected.

Vertical and horizontal transmission capabilities of SE, multiage flocks, poor biosecurity, rodents, darkling beetles, house flies, and improper composting are some of the ongoing challenges in controlling salmonellosis.

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RAPID TEST STRIP METHODOLOGY TO DETECT *SALMONELLA* GROUP-D_1 IN COMMERCIAL POULTRY PRODUCTION

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SUMMARY

The application of a commercial NPIP-approved lateral flow detection system using bacteriophages in enrichment media and monoclonal antibody to detect *Salmonella* Group-D_1 serotypes offers the advantages of lower cost, faster completion, high sensitivity, and specificity in comparison to the "gold standard" US-FDA BAM procedure. Laboratory evaluation and field application confirm the advantages of the technology in complying with statutory mandates and implementing control and eradication programs.

INTRODUCTION

To comply with Federal mandates and to implement an effective control program, a rapid screening procedure is required to detect the presence of *Salmonella* Group-D_1 serotypes with specific emphasis on *Salmonella* Enteritidis (SE). Samples which are routinely assayed comprise environmental drag swabs from litter and manure, exhaust fan dander, pooled egg contents, cloacal swabs, and carcass washing. Currently the US-FDA Bacteriological Analytical manual (BAM) procedure is recognized as the "Gold Standard" against which alternative identification procedures are evaluated for sensitivity, specificity, and accuracy. Due to the inherent disadvantages associated with traditional microbiological procedures, including time restraints, operator error, and cost, lateral flow test strips incorporating monoclonal antibody have been developed and approved for monitoring programs to control and eradicate Group-D_1 *Salmonella* serotypes.

The consequences of false negatives associated with suboptimal sensitivity or false positives due to low specificity are self evident. Undetected infections due to false negative assays may represent a risk to consumer health in the event of vertical transmission in eggs or on poultry meat. In addition, undetected infection will result in inevitable lateral transmission to other flocks by direct and indirect contact. False positive assays incur considerable expense associated with statutory responses, including re-testing flocks and products, possible depopulation, and enhanced

control measures which would otherwise be unnecessary.

LATERAL FLOW DETECTION SYSTEM

The *SDIX* RapidChek®SELECT™ SE detection system approved by FDA, NPIP, and AOAC, incorporates a two-stage enrichment over 48 h. The media used incorporate bacteriophages, which enhance selection by inhibiting bacteria present in the sample other than *Salmonella* Group-D_1. The detection process requires ten minutes after initiating flow on the test strip. In the event of a negative assay, the sample can be regarded as being free of *Salmonella* Group-D_1 and no further action is required. If a positive result is obtained, as denoted by two colored bands on the test strip, the sample is deemed "presumptive positive" and the status of the sample must be confirmed by following the BAM procedure.

The application of the *SDIX* RapidChekCONFIRM™ immunomagnetic separation test kit was developed to process "presumptive positive" specimens. High-affinity monoclonal antibody attached to magnetic particles selectively concentrates any *Salmonella* Group-D_1 which may be present, enhancing subsequent recovery using the BAM procedure.

SENSITIVITY AND SPECIFICITY OF THE RapidChekSELECT SE DETECTION SYSTEM

The following results were obtained under controlled evaluation:

Result	Group-D_1	*Non Group-D_1	Total
Positive	141	14	155
Sensitivity	100%		
Negative	0	196	196
Specificity	93%		
Total	141	210	351

*20 serotypes represented

Under practical conditions the following results were obtained by a commercial laboratory:

Number of drag-swab samples assayed =1,658

Number of presumptive positives using RapidChekSELECT SE=16

Number confirmed SE by BAM =1

This is contrasted with application of the BAM procedure:

Number of drag-swab samples assayed= 2,412

Number of samples yielding "colonies" requiring detailed examination =1,477

Number confirmed as SE by BAM=2

The additional labor, time and expense is evident in a comparison of the alternative detection methods.

It is emphasized that "presumptive positive" samples using the RapidCheckSELECT SE must be subjected to confirmation by traditional microbiology using the BAM protocol. In the case of the commercial laboratory, had this requirement not been followed, 15 presumptive positives would have been reported out as "positive" with obvious consequences.

It is estimated that the cost of a false positive result in a flock of 125,000 hens at 45 wk could amount to $74,500 as indicated:

COST CATEGORY	**COST PER FLOCK**
Sampling to 45 wk	$ 1,000
4 negative SE pools	$ 4,000
Loss on diversion over 4 wk	$66,500*
Cost of extra bio-security and disruption	$ 3,000
Total Cost of false positive	**$74,500**

*221,500 dozen @ 30cent differential between income from breaking compared with nest-run revenue of $1/per dozen

APPLICATION OF THE RapidChekCONFIRM IMMUNOMAGNETIC SEPARATION SYSTEM

In order to improve the rate of confirmation of presumptive positive results, evaluation trials applying the RapidChekCONFIRM system demonstrated the results in Table 1 when comparing RapidChekSELECT SE and BAM procedures to detect Group-D_1 in spiked manure drag swabs.

The application of the RapidChekCONFIRM imunomagnetic separation system enhanced recovery and confirmation of *Salmonella* Group-D_1, even in the presence of a Group-C_3 serotype (*Salmonella* Kentucky) using either lateral flow technology or a BAM protocol.

CONCLUSIONS

The NPIP-approved, *SDIX* lateral flow test system incorporating double enrichment and detection based on highly purified monoclonal antibody specific to *Salmonella* Group-D_1 offers the advantages of high sensitivity (100%) for screening the environment of flocks and poultry products. This allows rapid processing of large numbers of samples with quick turnaround to facilitate routine monitoring in addition to supporting control and eradication programs.

The high (93%), but not absolute specificity, allows differentiation of the target serotype from contaminants which may complicate interpretation of results using conventional BAM microbiology, which is time-consuming and subject to operator error.

For applications requiring extreme sensitivity for confirmation, the *SDIX* immune-magnetic separation can be applied before using either lateral flow technology or conventional microbiology

Immunomagnetic separation should be applied to complex samples such as environmental swabs in order to confirm the presence of SE. These specimens contain a diverse mixture of *Salmonella* serotypes other than SE requiring concentration before applying BAM procedures to avoid "picking" a large number of colonies for microbiological examination.

Table 1. Confirmation of presumptive positive results comparing RapidChek®SELECT™ SE and BAM procedures.

Detection Procedure	Spike	No. samples examined	No. presumptive positive	No. with Group-D_1	
				Before IMS	**After IMS**
RapidChek	None	65	1	0	0
	SE	10	10	10	10
	SE+SK	10	10	4	10
	Total	**85**	**21**	**14**	**20**
Conventional BAM	None	5	3	0	0
	SE	10	10	9	10
	SE+SK	10	10	2	9
	Total	**25**	**23**	**11**	**19**

FALSE POSITIVE IMMUNOASSAY RESULTS DUE TO SE KILLED VACCINE

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As part of the Food and Drug Administration (FDA) egg rule, drag swabs are collected and tested for the presence of *Salmonella* Enteritidis (SE) (2). The first collection is done when the pullet flock is between 14 to 16 weeks old. Drag swabs are also collected when layers are 40 to 45 weeks of age and also four to six weeks after molt. Detection of SE from environmental samples may be done by traditional cultural methods, by the SDIX RapidChek® SELECT™ or Neogen Reveal SE test systems (3). Cultural methods have a turnaround time of 5 to 10+ days, while the latter two have a turn around time of 48 hours.

Both of these rapid test systems detect antigens of any *Salmonella* spp. belonging to group D and not just SE. They cannot differentiate between the antigens of live bacteria or just the presence of bacterial wall antigens from dead bacteria or bacterial fractions. For these reason, the Avian Health and Food Safety laboratory (AHFSL) reports positive results from the rapid test system as "presumptive" and only samples confirmed by culture are reported as positive.

In 2011 a total of 1,162 environmental samples were tested using the SDIX RapidChek system at the AHFSL. Twenty of those drag swabs have been reported as presumptive. Of these 20 samples only two were confirmed positive SE by culture. Interestingly 15 of the other 18 presumptive samples were from three independent cases submitted by different companies in which either non-group D or no *Salmonella* was detected by culture. In all these cases, the pullets had been vaccinated with an SE bacterin two to five days prior to the collection of the drag swabs. It was hypothesized that the SDIX RapidChek test system was detecting the bacterin that had spilled in the house during vaccination.

To test this hypothesis, Hydra sponges (3M, St Paul, MN) and 10 x 10 inch sterile gauze pads moistened with evaporated milk were spiked with 0.25, 0.5, 1, 2, and 3 mL of bacterin. The samples were set up and incubated as per the SDIX RapidChek protocol (1). SDIX stick tests showed positive results in swabs that had had been spiked with 0.5 mL or more of the bacterin in the hydra sponges and 0.25 mL in the gauze pads.

Although vaccination for SE is not required by the FDA rule, many producers use SE vaccines to aid flocks in remaining SE negative and for eggs to test negative for SE. Live vaccines are attenuated *S.* Typhimurium (serogroup B), while killed vaccines or bacterins contain SE antigens. Bacterins are given at 13 to 15 weeks of age. Environmental samples are collected between 14 to 16 weeks of age from the pullet houses. Because of the short time between vaccination and collection of drag swabs in pullet houses, it is possible that residual bacterin is present in different areas of the house where environmental samples are collected. Laboratories using only one of the rapid detection systems may find positive environmental samples that contain only bacterin and not actual live SE.

In summary, the SDIX RapidChek is a sensitive and rapid method, but it can detect bacterin or dead SE present in an environmental sample. Even though may not be required under the FDA egg rule, isolation of *Salmonella* and confirmation of live SE should be performed on samples that are positive by a rapid test to avoid false positive results.

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

LIVE ATTENUATED *SALMONELLA* GALLINARUM VACCINES FOR POULTRY

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SUMMARY

Salmonella Gallinarum is the causative agent of fowl typhoid and remains an economical disease of poultry in developing nations (1, 5, 6). An ideal vaccine for fowl typhoid should ensure safety in birds of all ages, easy to administer, cost-effective, and highly immunogenic in few doses. Regulated delayed attenuation strategy using conditional mutations affecting O-antigen synthesis and virulence genes in *Salmonella* Typhimurium are impaired in virulence and protective in mice (2-4). In order to construct safe and highly efficacious vaccines for fowl typhoid, we constructed a series of conditional mutants affecting O-antigen synthesis and virulence genes in the sequenced strain of *Salmonella* Gallinarum. Such mutants, when grown in presence of sugars, produce smooth, full length LPS and express wild-type genes required for virulence or colonization. After several generations of growth inside host, the vaccine switches into rough form or turn off virulence genes, in the process, generating attenuated strain. To evaluate protective efficacy of vaccine candidates, an oral challenge model for fowl typhoid was developed and ten point scoring system was created to assess health of birds post-vaccination or challenge. We screened a strain, χ11422, which requires mannose and arabinose for expression of O-antigen and an arabinose dependent *crp* strain, χ11387. Both χ11387 and χ11422 is highly attenuated and protective against a lethal oral challenge of S. Gallinarum (Table 1). These orally administered vaccines are easy to administer and cost-effective.

(The full length article “Safety and protective efficacy of live attenuated *Salmonella* Gallinarum vaccines in Rhode Island Red chickens” will be submitted to the *Avian Diseases*.)

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Table 1. Protective efficacy of chicks vaccinated with *S*. Gallinarum mutants post challenge.

Strain	Genotype	Alive/Total (Post challenge)		
		Expt. 1	Expt. 2	Expt. 3
BSG	-	1/5	0/5	2/10
χ11387	ΔPcrp527::TT *araC* P_{BAD} *crp*	4/5	5/5	10/10
χ11422	Δ*pmi-2426* $\Delta P_{rfc\ 174}$::TT *araC* P_{BAD} *rfc*	5/5	5/5	10/10

PRELIMINARY ANTIMICROBIAL RESISTANCE RESULTS IN A GAME BIRD COMMODITY (BLACK SILKIE CHICKEN) AND REVIEW OF ANTIMICROBIAL THERAPY IN MINOR SPECIES OF POULTRY IN CANADA

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ABSTRACT

Available veterinary antimicrobials in Canada are labelled for the therapy of common bacterial diseases in major food animal species, e.g., chickens, pigs, cattle, and turkeys. In poultry, antimicrobials are usually indicated for the therapy of major diseases affecting chickens and turkeys, but not for less frequently occurring and emerging conditions (minor use) or for use in minor poultry species or "alternative poultry species." These poultry types contribute to the emerging "niche market" production in Canada and are non-regulated, i.e., not controlled by a provincial or national marketing board. This paper provides an overview of antimicrobial use (AMU) practices in minor species of poultry and preliminary results of a pilot project on antimicrobial resistance (AMR) in Black Silkie chickens, as one species in the game birds population in Canada.

INTRODUCTION

Consumer perception and increasing food quality and safety awareness have influenced farming practices, i.e., switching from conventionally raised to antibiotic free or organic production. Diversity and continuous exploration of non-traditional meats (i.e., not chicken or turkey) have also influenced the range of poultry products available to consumers. As a result, minor poultry production has increased in the last decade in Canada (1). Some of the minor species of poultry available for consumption in Canada include domestic ducks and geese, ratites (ostrich and emus), quail, guinea fowl, pheasants, pigeons, Black Silkie chickens, and other "game" birds. These species are

sometimes considered "alternative species" or "niche market" commodities intended for some restaurants and certain ethnic groups. The overall population of minor species is relatively low.

In Canada, most of the veterinary antimicrobials available are generally labelled for the therapy of major bacterial diseases affecting major animal species, such as chicken, cattle, pigs and turkeys. The Veterinary Drugs Directorate sets Maximum Residue Limits (MRL) to ensure safety of antimicrobials (from the residue as a chemical hazard) to both animals and people when these antimicrobials are used in animal production (6). Except for few data in ducks (e.g., neomycin), MRL data for minor species are generally unavailable; hence, approved drugs have limited indications, i.e., for use only in major species to treat commonly occurring or major infections. The limited therapeutic options may pose a challenge for producers and veterinarians in the face of an outbreak. Legislations regarding minor use and minor species (MUMS) are available in other countries to address the need for therapeutic products. Some examples of MUMS regulations in existence are:

- **United States**: MUMS Animal Health Act (2004). Aimed to address severe shortage of approved new animal drugs for use in minor species and in treating animal diseases and conditions that occur infrequently or in limited geographical areas, cost and economic considerations for pharmaceutical companies, and to avoid lengthy approval processes for new drugs (5).
- **Europe:** Policy for Classification and Incentives for Veterinary Medicinal Products Indicated for Minor Use Minor Species/Limited Markets. Aimed to encourage pharmaceutical companies for the development of new veterinary medicines for MUMS (4).
- **Australia:** Agricultural and Veterinary Code Regulations 1995 (Minor Use). Allows the issuance of permit for use in minor food-producing species where the economic return would not be sufficient to meet the cost of registration of the veterinary product (2, 3).
- **Canada:** No MUMS policy in existence, but government-industry consultations to develop MUMS policy/regulation is underway (7).

Surveillance of clinically-relevant organisms in chicken layers, domestic ducks, and other minor poultry species are not yet covered by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) or any government monitoring program, but targeted research and pilot projects have been conducted to assess levels of AMR contaminated bacteria in selected poultry species. This paper provides an overview of antimicrobial use (AMU) practices in minor species of poultry and preliminary results of a pilot project on antimicrobial resistance (AMR) in Black Silkie chickens, as one species in the game birds population in Canada.

MATERIALS AND METHODS

AMU: Minor diseases of major species of poultry (i.e., layer hens) and minor species of poultry were obtained from published Canadian case reports and laboratory newsletters. Literature searches of *in vivo* (animal/clinical trials, pharmacokinetic studies) and *in vitro* (based on antimicrobial susceptibility testing methods) were also conducted using electronic databases and poultry-specific online journals from January to December 2011. Antimicrobials were grouped according to their importance to human medicine, using the categorization system of Health Canada's Veterinary Drugs Directorate (VDD) as follows: Category I - Very High Importance; Category II - High Importance; Category III - Medium Importance, and Category IV - Low Importance (8). The Canadian Association of Veterinary Medicine Prudent Use Guidelines (CVMA-pug) 2008, the 2009 Compendium of Veterinary Products (CVP), and the Compendium of Medicating Ingredient Brochure (CMIB) online were reviewed to determine applicable guidelines and policies regarding veterinary antimicrobial use in Canada.

AMR: Whenever available, a maximum of three Black Silkie Chickens (whole bird carcass) were purchased from major urban areas in two provinces included in the retail component of the CIPARS program: Ontario (Toronto) and British Columbia (Vancouver, Victoria). Details regarding methods used for retail sample collection, culture, and antimicrobial susceptibility testing are described in the CIPARS annual reports (9).

RESULTS AND DISCUSSION

Diseases and antimicrobial use practices. Surveillance programs with animal health themes are rare in Canada, but few diseases in major species of poultry, such as layers, have been reported. These reported diseases include *Brachyspira* sp., a spirochete associated with dirty table eggs in Ontario, and *Mycoplasma*, an organism associated with respiratory diseases (infectious sinusitis). Diseases of minor species such as *Riemerella anatipestifer* (ducks), *Chlamydophila* (pigeons), and *Salmonella* (pigeons, ratites) have also been reported. However, current prevalence of these diseases in Canadian poultry and AMU practices are largely unknown. Antimicrobials

identified in the literature search for use for minor poultry species are summarized by VDD Category in Table 1. The contributing studies were conducted in different countries investigating the susceptibility patterns (*in vitro*) of isolates, field observations, or efficacy studies. Overall, the number of available literature sources was very limited (n=15). Antimicrobials included in Table 1 are available in Canada, but would have to be used in an extra-label drug use (ELDU) manner because of their current approval and marketing status. Few drugs have indications for the therapy of infections in leghorn chickens at the laying phase (e.g., *Mycoplasma*-associated). Over-the-counter antimicrobials are also generally labelled for use in chickens and turkeys but not for use in laying hens and minor poultry species. In the absence of MUMS acts/regulation in Canada, the Veterinary Drugs Directorate addresses veterinary issues pertaining to MUMS on a case by case basis (Dr. Manisha Mehrotra, personal communication).

Bacterial recovery rates. (Note: bacterial recovery rate and AMR *data are preliminary (Nov. 2010 – early Oct. 2011), more complete, up-to-date data will be presented at the conference*) Recovery rates of bacteria from Black Silkie chicken in 2010-2011: *E. coli* - 98% (51 isolates/52 samples), *Salmonella* - 15% (8/52), and *Campylobacter* - 46% (24/52). To-date, thirty two of the *Salmonella* isolates were serotyped and phage typed. The serovars detected were: Typhimurium PT 132 (3/8, 38%), Typhimurium var. Copenhagen (2/8, 25%), and one isolate each of Heidelberg, I:6,7:k:-, and Kentucky. The *Campylobacter* species detected were *C. jejuni* (18/24, 75%) and *C. coli* (5/24, 21%); one isolate was unspecified (1/24, 4%).

Antimicrobial resistance.

E. coli. No resistance to ciprofloxacin or ceftiofur (both VDD Category I drugs), was detected, but resistance to ampicillin (2/13, 15%), streptomycin (4/13, 31%), sulfisoxasole (1/13, 8%), and tetracycline (5/13, 38%) was observed.

Salmonella. Of the five isolates that were susceptibility-tested as of Jan. 9th, 2012, resistance to amoxicillin-clavulanic acid (2/5), ceftiofur (1/5), ampicillin (3/5), streptomycin (3/5), chloramphenicol (2/5), sulfisoxasole (2/5), and tetracycline (3/5) was detected.

Campylobacter. Of the 16 isolates that were susceptibility tested as of Jan. 9th, 2012, two (13%) isolates were resistant to both ciprofloxacin and nalidixic acid and were recovered from black silkie chicken samples purchased in Ontario. Six (38%) isolates were resistant to tetracycline.

CONCLUSIONS

Few antimicrobials are available for treating minor infections of major poultry species such as chicken laying hens, and no labelled antimicrobials are available for minor poultry species. Preliminary results of a retail pilot project investigating AMR in select bacteria from Black Silkie Chickens reveal that resistance to various antimicrobials have emerged in the Canadian game bird population. Of note with respect to public health, is resistance to ciprofloxacin and nalidixic acid in two *Campylobacter* isolates from meats purchased in Ontario. Also of note is resistance to antimicrobials proposed in the literature for use in minor poultry species. Antimicrobial use practices are unknown in poultry in Canada. CIPARS will continue to monitor AMR in Black Silkie Chickens to assess the impact of niche market production in the epidemiology of AMR in Canadian poultry and potential human health implications.

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Table 1. Literature review of antimicrobial[a] therapy of minor diseases of chicken laying hens and minor poultry species.

	***Brachyspira* spp.**	***Mycoplasma synoviae***	***Riemerella anatipestifer***	***Chamydophila psittaci***	***Salmonella* Typhimurium**	
Type of use	Minor use	Minor use	Minor species	Minor species	Minor species	Minor species
Avian Type	Chicken egg layers	Chicken egg layers	Ducks, Geese	Pigeons	Pigeons	Ratites
I[b]			*Ceftiofur* (SC[d] only), Rx *Enrofloxacin*, Rx			
II	*Lincomycin* *Tiamulin* *Bacitracin (Zn)*	Tylosin	*Ampicillin*, Rx *Erythromycin* *Lincomycin-spectinomycin* *Neomycin* *Neomycin-oxytetracycline* *Ormetoprim-sulfadimethoxine*, Rx *Penicillin G* *Tylosin*		*Amoxicillin*, Rx	
III		Chlortetra-cycline	*Florfenicol*, Rx *Sulfaquinoxaline* *Sulfamethazine*	*Tetra-cyclines*		*Oxytetra-cycline*
IV						
Unknown			*Novobiocin*			

[a] Cited by various authors and are available in Canada for veterinary use. Italicized antimicrobials are used in an extra-label manner for indications, species, or dose.

[b] Roman numerals I to III indicate the ranking of antimicrobials based on importance in human medicine as outlined by the Veterinary Drugs Directorate, Health Canada.

[c] Rx - prescription only recommended by manufacturer.

[d] SC – subcutaneous route; *in-ovo* route of administration is not indicated for this product.

DIETARY FEED ENZYME AND BIOTIN SUPPLEMENTATIONS DO NOT AMELIORATE FOOT PAD DERMATITIS IN BROILER CHICKENS

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SUMMARY

Four broiler chicken experiments (Exp.) were conducted to evaluate effects of dietary enzyme and biotin supplementation on the development of footpad dermatitis (FPD) on replicated floor pens. Although enzyme supplementation of diets reduced intestinal viscosity, no effect on ammonia volatilization or the incidence and severity of FPD was detected. Likewise, FPD was not affected by biotin supplementation. The FPD incidence and severity did not correlate with enzyme or biotin supplementation but appeared to correlate more with high litter moisture levels.

INTRODUCTION

The incidence and severity of FPD in broiler chickens is of great concern to the broiler industry, not only from a product quality but also from animal welfare standpoints. The etiology of FPD points to a complex interaction of various risk factors, such as genetics, market weight, bedding type and litter quality, flock health, nutrition, feeding, and management programs (1, 2). FPD have been reported in birds fed high nutrient density feeds, high protein feeds and feeds formulated with all-vegetable ingredients (3, 4, 5). Simple and effective modifications to nutrition and feeding programs are frequently sought by nutritionists to reduce the incidence and severity of FPD in commercial flocks. The use of enzymes targeting the non-starch polysaccharides (NSP) component of the diets is often considered in an effort to reduce excreta viscosity and to minimize foot pad irritation from prolonged fecal adherence (6, 7). Likewise, supplementation of diets with higher levels of biotin have also been suggested to reduce ulceration and hyperkeratinization of the feet and shanks often observed with the deficiency of this vitamin (8, 9).

MATERIALS AND METHODS

The effect of feed-grade commercial enzymes (control plus six enzyme treatments to 49 d of age in Exp. 1, and control plus four enzyme treatments to 56 d of age in Exp. 2) on FPD were evaluated with corn-soy based diets in replicated floor pens (eight replicate pens of 24 male chicks per treatment in Exp. 1, and six replicate pens of 45 straight-run chicks per treatment in Exp.2. In Exp. 3 and 4, the influence of four levels of dietary biotin (0.05, 0.1, 0.2, 0.4 mg/kg) on FPD (10 replicate pens of 15 chicks per treatment to 57 d of age in Exp.1 and eight replicate pens of 45 chicks per treatment to 56 d of age in Exp.2). In addition to live performance (weight gain, feed conversion, and mortality) the incidence and severity of FPD was scored (based on a three point scale) in all birds. Intestinal viscosity (on d 15, 36, and 50 in Exp. 1) and ammonia volatilization (on d 42 in Exp. 1) were also measured. Litter moisture was determined in Exp. 1, 2, and 4.

RESULTS AND DISCUSSION

The live performance of birds did not vary among the dietary treatments in either enzyme experiments ($P>0.05$). Enzyme supplements did not affect the incidence and severity of FPD or ammonia volatilization significantly. However, birds reared on enzyme-supplemented diets had lower ($P<0.001$) intestinal viscosity levels in Exp. 1. Likewise, no differences in live performance or the incidence and severity of FPD was detected with different dietary biotin levels in Exp. 3 and 4 ($P>0.05$). FPD increased when litter moisture >30%. Litter moisture exceeded 35% in all treatments by d 56 in Exp. 4.

Results of this study indicate that enzyme supplementation of corn-soy diets results in a significant decrease in intestinal viscosity, with no effect on FPD. The FPD incidence and severity appeared to correlate more with high litter moisture levels. Recent observations indicated that FPD may be initiated early in the grow-out and that subsequent improvements in litter quality may help reverse the

lesion severity, as observed in this study (Figure 1). Flock management programs (i.e., bedding type, particle size and depth, litter moisture, and house ventilation) appear be the primary drivers for this contact dermatitis in poultry.

(The full length article will be published in *British Poultry Science*.)

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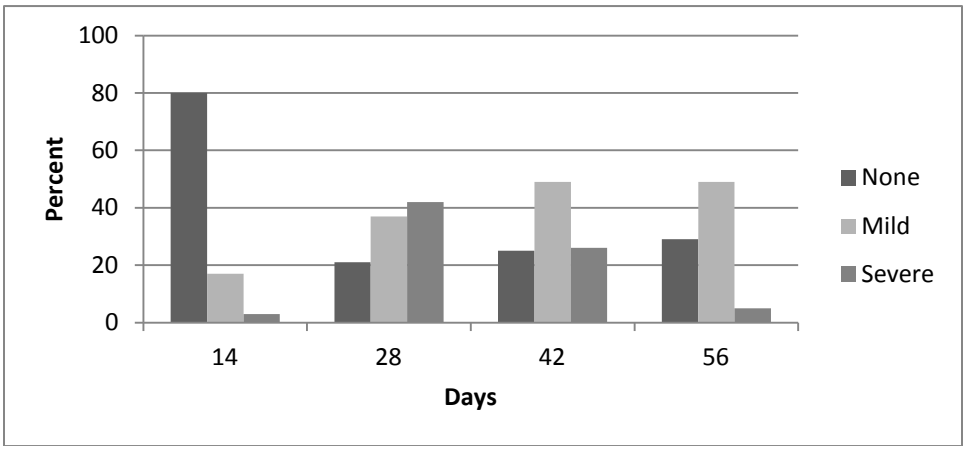
Figure 1. Effect of flock age on FPD incidence and severity* (Exp. 4).



* None=No lesions; Mild=lesions <7.5 mm; Severe=>7.55 mm

EVIDENCE OF *MYCOPLASMA IOWAE* IN GAME BIRD FLOCKS

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ABSTRACT

Game birds are avian species bred for hunting purpose. There is a lack of knowledge regarding the presence of specific pathogens in this sector. As the game birds are usually released in the wild, their "unknown" pathogens could represent a threat for the poultry industry. *Mycoplasma iowae* (MI) is considered a pathogen of turkeys, even though it has been isolated in different avian species. It has been demonstrated that MI shows a preference for the gastrointestinal environment, probably related to its ability to grow in the presence of bile salts. This is considered an unusual property for avian mycoplasma. The aim of this report is to describe the first detection of MI in some groups of pheasants, grey partridges, and chukars, which showed a similar history of intestinal and respiratory disorders.

INTRODUCTION

MI is considered one of the most important mycoplasma species involved in poultry disease. Its pathogenic role has been mainly demonstrated under experimental infections. The most important clinical sign was an increase of embryo death in turkeys and chickens. The infected pullets showed poor feathering, scarce growth performance, and skeletal abnormalities. Airsacculitis is not a typical sign and if present it is usually mild.

As MI has preference for the gastrointestinal tract, the cloacal and phallus swabs are considered the best choice for sampling. The tropism for the intestinal tract is considered unusual property for avian mycoplasma except for *Mycoplasma meleagridis*, which could be easily isolated by cloacal swabs. The ability of MI to grow in the presence of bile salts confirms its adaptation to intestinal environment.

MI has been demonstrated in a large amount of wild avian species even if no specific clinical signs were reported.

Game bird breeding is considered a relative important sector in Italy. Birds species involved in this type of production are pheasants, grey partridges, and chukars. These birds are reared both indoors and outdoors until five to seven months of age and then are released in the wild in order to increase the wild stock for hunting purpose.

During the indoor phase, respiratory and intestinal disorders can affect the birds stock. The main pathogens involved are *Aspergillus* spp., *Escherichia coli*, and *Mycoplasma gallisepticum* for the respiratory tract whereas coccidia, *Trichomonas* spp. *and Clostridium* spp. for the intestinal tract.

In this report the first isolation of MI in groups of game birds is described.

CASE REPORT

In the spring and summer of 2011, several groups of pheasants, grey partridges, and chukars were submitted to the Diagnostic Unit of Istituto Zooprofilattico Sperimentale delle Venezie for a necropsy examination. The farmers reported a common history of scarce state of nutrition, poor feathering, increased mortality, increased number of culled birds, soiled vent, and in some cases, a bilateral sinusitis.

MATERIALS AND METHODS

About 40 carcasses, around one to three months of age, were submitted and examined in the necropsy room of the Institute. The same diagnostic protocol was applied to all birds, consisting of gross pathologic examination, virological tests (infectious bronchitis, Metapneumovirus, and transmission electron microscopy of the gut), bacteriological, and parasitological tests. Samples collected from infraorbital sinus and cloaca were submitted for *Mycoplasma* spp. isolation according to the internal protocol. The identification of suspect colonies was performed by different techniques, including immunofluorescence (IFAT), indirect immunoperoxidase test, denaturing gel gradient electrophoresis (DGGE) (2), and confirmed by sequencing of 16S gene (1).

RESULTS AND DISCUSSION

The birds submitted for the necropsy showed a scarce state of nutrition, a soiled vent area and bones fragility. Moreover, enteritis was detected, and in some birds, presence of airsacculitis and sinusitis. No significant differences were noticed among the three avian species analyzed.

Virological test results were negative, whereas bacteriology showed *Escherichia coli* from gut and

sinus. The majority of birds showed an infestation of coccidial and *Trichomonas* species. Mycoplasma isolation showed the presence of typical colonies in the infraorbital sinus and cloacal swabs. The colonies were identified as MI through the immunological and the biomolecular methods described above.

MI was detected mainly in gut samples. Some specimens collected from inflamed sinus were positive as well.

Our results suggest that pheasants, grey partridges, and chukars are susceptible to MI infection. MI seems to be responsible for a nonspecific disease with a scarce state of nutrition, poor feathering, and bone fragility. These clinical and pathology findings are relatively similar to the ones observed in turkeys infected with MI. It is important to consider that co-infection with protozoa could exacerbate the gut disorder, making it more difficult to identify the true pathogenic role of MI in these avian species.

Further studies are recommended to clarify the role of MI in game birds in order to elucidate its potential epidemiological role in the spread and transmission of this pathogen to poultry industry

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OUTBREAKS OF MASSIVE RED MITE INFESTATIONS IN CHICKENS

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ABSTRACT

The red mite (*Dermanyssus gallinae*) is a common ectoparasite affecting poultry productions worldwide. Two fatal outbreaks of mortality due to red mite massive infestations are described affecting commercial hens from a medium size farm and backyard broilers. Extremely severe anaemia was the main feature in both cases.

INTRODUCTION

The red mite, found worldwide, is the most economically deleterious parasite of laying hens in Europe (1). It is an obligatory hematophagous ectoparasite of both domestic and wild birds, and is known to obtain its blood-meal on a range of alternative species, including man. The poultry red mite is considered a temporary parasite since its life cycle is spent mainly in the environment (cracks and crevices of the poultry facilities, cages, perches, etc.) reaching the host only for feeding. A blood feeding takes one to two h and usually takes place after sunset or at night (2). This mite affects both poultry health and welfare. The red mite causes irritation, restlessness, and anemia, and can also evoke cannibalistic behavior (i.e. feather pecking).This results in severe economic losses, especially in layer farms. Control measures are extremely difficult because red mite populations increase quickly in numbers diffusely occupying poultry facilities (3).

MATERIALS AND METHODS

Two outbreaks out of our caseload have been chosen as representative of red mite infestations during summer, both in commercial layers and backyard chickens.

Layers. Two 50 wk birds from a multiage, medium-size farm with increased mortality localized mainly in the cages of the central rows. These birds were alive on arrival and were humanly sacrificed.

Backyard broilers. Five four wk old colored birds from a family poultry farm where similar cases of mortality had been observed few days earlier.

Submitted birds were necropsied. Blood smears from the cardiac chambers were obtained from the backyard broilers. The smears were stained routinely with Hemacolor. Parasites were collected for microscopic examination.

RESULTS

At necropsy, both layers and backyard broilers had myriads of red mites on skin and feathers. Parasites were alive and moved actively on the necropsy table.

Layers. The two birds showed good body condition, extremely pale combs and carcasses, forming eggs in the oviducts.

Backyard broilers. All the birds showed normal amounts of food in their crops and gizzards. They had severely pale carcasses and bone marrow. Hypoplasia of bursa of Fabricius and thymus was also observed. Blood smears were characterized by high numbers of variably immature red cells.

DISCUSSION

In Italy, red mite is the most common ectoparasite affecting poultry productions. Nevertheless, this infestation is rarely taken into account in cases of increased mortality and is often underestimated. Surprisingly, such massive red mite infestations were missed and/or neglected by farmers who did not even mention it in the anamnesis. This attitude is strikingly exampled in the outbreaks described where the infestation severity was due to the lack of any control measure. Red mite control is actually difficult but no control leads to increasing the economic losses and numbers of fatal cases.

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ESTIMATION OF THE EFFICACY OF A CHEMICAL AND A BIOLOGICAL LITTER AMENDMENT IN REDUCING AMMONIA LEVELS, BACTERIAL LOAD, AND PAW LESIONS IN COMMERCIAL BROILER FACILITIES

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SUMMARY

Six commercial broiler houses (12.2×152.4 m) were monitored for three consecutive flocks to observe the effect of a biological litter treatment (Litter Guard: LG) or chemical litter treatment (Poultry Litter Treatment: PLT) on ammonia volatilization, litter pH, moisture, and microbial load. Three houses were treated with LG seven d before bird placement; 18.9 L of LG in 378.5 L of water applied to the entire floor area. The other three houses were treated with PLT 24 h before bird placement applied only in the central brooding area at the rate of 24.4 kg/100m^2. Litter samples were analyzed for pH, moisture, and microbiological analysis, which included determination of total *E. coli*, coliform, and *Clostridium perfringens* counts (CFU/g). A Drager CSM analyzer was used for ammonia measurement. Five hundred paws were assessed from each house for all three flocks at processing. PLT significantly (P<0.10) reduced ammonia levels on d one of sampling as compared to LG (37.9 vs. 59.4 ppm), whereas LG gave a significantly (P<0.10) lower ammonia value on d 15 as compared to PLT. For PLT treated houses a lower pH for all three flocks on d one was measured as compared to LG treated houses. Litter moisture and microbial counts (CFU/g) did not show any significant differences (P>0.10) throughout the trial. The PLT treated houses showed a significantly (P<0.10) higher incidence of affected paws throughout the trial.

INTRODUCTION

Increased ammonia levels in commercial facilities create a stressful ambient environment for broilers and this environment may contribute to immunosuppression, respiratory disease, increased susceptibility to viral and secondary bacterial infections, and severe impact on production. Moore et al. (2) demonstrated the use of different litter amendments in reducing ammonia volatilization from poultry litter. Increased bacterial load in litter is

concerned with increased chances of infection to birds. PLT has proven to decrease ammonia emission rates, pH, and bacterial load in litter (3). PLT also helps to delay the onset of campylobacter colonization in broiler chicks, thus effectively reducing severe consequences of this pathogen (1). PLT also contributes to a reduction in the incidence of ascites in broilers (5). This current trial was a comparative study to notice the effect of PLT and LG (newly introduced microbial litter amendment) on ammonia emission rates, litter pH, bacterial load, and paw quality in commercial broiler facilities.

MATERIALS AND METHODS

A total of six commercial broiler houses (12.2 x 152.4 m; 43 x 510 ft) were used in this field trial to evaluate the effectiveness of LG and PLT as a litter additive in controlling ammonia volatilization, microbial growth, and litter quality. Three consecutive grow outs were monitored. Three houses were treated with LG seven d before bird placement; 18.9 L of LG in 378.5 L of water (5.28 gallons into 100 gallons water) applied to the entire floor area. The other three houses were treated with PLT 24 h before bird placement applied only in the central brooding area at the rate of 24.4 kg/100m^2 (50 lbs/1,000 ft^2). Number of birds placed in each house was 28,000 head. All samplings were obtained initially and weekly during the six to seven wk trial period from four permanently identified locations within each quadrant of each house.

Ammonia measurements were conducted using a closed container of specified dimension (53.3 x 39.4 x 12.7 cm) inverted over the litter bed and determined using a Drager CMS Analyzer (Drager, Pittsburgh, PA) equipped with a remote air sampling pump and appropriate ammonia sampling chip (0.2-5, 2-50, or 10-150 ppm). The tube from the sampling pump was located in the top center of the container. The sampling pump was evacuated (calibrated) for 60 seconds followed by a measurement period of up to 300 seconds. Most readings were achieved within 60 seconds following evacuation. Litter pH was determined by taking 5 g of litter from each sample and adding 45 mL of distilled water to it. This litter water mixture was then thoroughly mixed and allowed to sit for 45 min. After which time the pH was determined using an electronic pH meter.

Microbiological analyses included total *E. coli*, coliform and *C. perfringens* counts (CFU/g). All bacterial colonies were enumerated and the amount of bacteria per g was calculated and averaged for each treatment. At processing, 500 paws were collected from each house and visually scored for quality. Statistical analyses were performed on all data using JMP® software (4). Data were subjected to a t-test analysis at a probability level of 0.10.

RESULTS AND DISCUSSION

PLT significantly ($P<0.10$) reduced ammonia levels on d one as exhibited to LG (37.9 vs. 59.4) but was not effective in maintaining the ammonia levels as LG lowered readings after d one of trial. Litter pH was significantly ($P<0.10$) lowered by PLT as compared to LG on d one (7.91 vs. 8.43) and d 29 (8.22 vs. 8.36), otherwise pH levels were similar for both treatments. Total *E. coli* counts did not show any significant ($P>0.10$) results throughout the trial. Total coliform counts also did not show any significant ($P<0.10$) differences but there was a gradual increase in total coliform count with increasing age of birds until 36 d. Litter treatments did not have any significant ($P>0.10$) effect on total *C. perfringens* count for the entire trial except on d 43 where PLT showed significantly higher counts than LG.

The PLT treated litter showed 22.2% (1009) of the total paws of score one (mild dermatitis) much higher than LG treated litter with 15% (690) paws with score one (Table 2). PLT treated litter also showed large number of score two (severe dermatitis) as compared to LG treated litter (4.3% vs. 0.9%). Thus PLT proved to influence a decline in the paw quality of birds as compared to the LG treatment. LG proved to be effective in reducing the ammonia emission rates significantly for most of samplings as compared to PLT. PLT on the other hand was efficient in reducing pH reading for most of samplings. Microbial counts were not significantly affected by either of the treatments. LG also reduced the incidence foot pad dermatitis.

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on death due to ascites in broilers. Avian Dis. 42:385–387.

Table 1. Effect of LG and PLT on litter parameters (Trials 1-3) [1].

	Age (days) [2]							
	Initial	**1**	**8**	**15**	**22**	**29**	**36**	**43**
Ammonia (ppm)								
LG	54.5[b]	59.4[a]	39.8	34.2[b]	63.7	114.8	100.4[b]	104.1
PLT	78.0[a]	37.9[b]	41.8	41.4[a]	70.3	118.0	120.3[a]	109.4
pH								
LG	8.36	8.43[a]	8.18	8.01	7.95	8.36[a]	8.26	8.28
PLT	8.39	7.91[b]	8.16	8.00	7.88	8.22[b]	8.23	8.31
E.coli count (cfu/g)[3]								
LG	3.905	3.404	4.823	5.586	5.476	5.136	4.810	4.325
PLT	3.709	3.548	4.722	5.389	5.328	5.163	4.744	4.279
Coliform count (cfu/g)								
LG	3.899	3.820	4.977	5.818	5.534	5.356	5.439	4.717
PLT	4.024	3.777	5.029	5.525	5.517	5.370	5.546	4.676
Clostridium perfringens (cfu/g)								
LG	3.982	3.949	4.007	4.250	3.916	4.433	3.748	3.646[b]
PLT	3.975	3.876	4.089	4.256	3.967	4.450	3.765	3.812[a]

[1]Values are grand means derived from six commercial broiler houses (three per treatment level) sampled at four specified locations within each house.
[2] Age of birds and day of sampling. The days indicated are for Trial 1. Sampling days for Trial 2 and 3 (here indicated as Trial 1 and 2) were 1, 8, 15, 22, 29, 36 and 45 and 4, 11, 18, 25, 32, 39 and 46, respectively.
[3]cfu/g = colony forming units per gram of litter sample.
[ab] Numerical values for each variable column with different superscripts are significantly different at P<0.10.

Table 2. Effect of LG and PLT on paw quality scores.

	Score*		
Treatment	**0**	**1**	**2**
LG	3859 (84%) **	690 (15%)	43 (1%)
PLT	3335 (73%)	1009 (22%)	199 (5%)

*Score 0 = no lesions on foot pad, Score 1 = mild dermatitis lesions, Score 2 = severe dermatitis lesions.
**Figures in brackets represent the percentage of score of the total scores for each treatment individually.

TOXICITY INDEX PROPOSAL TO BETTER EXPLAIN MYCOTOXIN INTERACTIONS

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SUMMARY

Past research focused on the effects of exposure to a single mycotoxin in poultry feed while recent studies have demonstrated interactions occur when two different mycotoxins are present. Such interactions can lead to toxicity at mycotoxins concentrations at which no toxicity is expected when evaluating each mycotoxin in isolation. In the real world, evaluation of the toxicity of feed often encounters three, four or more different mycotoxins in the same feed. How is it possible to estimate the potential toxicity of a feed considering multiple mycotoxins and their interactions? This paper proposes that a "Toxicity Index" (TI) can be developed for each mycotoxin present in the feed based on published LD_{50} values. The TIs are calculated using the ratio of LD50 value of the least toxic mycotoxin by the LD50 value for each of the other mycotoxins identified. Each TI is then multiplied by the corresponding mycotoxin concentration in the feed.

The products of such multiplication are summed to obtain a total mycotoxin concentration which can then be used to predict the potential toxicity of the contaminated feed.

Mycotoxins are the secondary toxic metabolites produced by various genera of molds. Molds that produce mycotoxins of significance in poultry industry include *Aspergillus, Fusarium*, and *Penicillium*. Though hundreds of mycotoxins have been implicated in animal disorders, the most significant for poultry industry include aflatoxins, ochratoxins, T-2 toxin, deoxynivalenol (DON), fumonisins, and zearalenone (ZEA) (Devegowda and Krishnamurthy, 2005). Commonly two or three mycotoxins are analyzed in poultry feed based on the geographical region.

Since one mold can produce several mycotoxins and several molds can be present in one feedstuff, it is expected that there are likely more mycotoxins present than are being tested. To give an example, if a sample contains T-2 toxin there are chances for the presence of several other mycotoxins with the predominant ones being HT-2 toxin, and neosolaniol. As these toxins contribute to the toxicity of T-2 toxin, the lack of analysis and not considering their toxicity will give a false sense of security. The same situation applies for the various forms of aflatoxins, DON-related compounds, fumonisins and ergot toxins. As a result, analysis of as many toxins as possible at an affordable cost and in a timely manner will assist in a better prediction of toxicity to animals and the development of preventive solutions in situations of multiple mycotoxins.

The difficulty is how to interpret the total toxicity to poultry if more than one mycotoxin is detected in poultry feeds. Studies have been conducted in poultry assessing mycotoxin interactions when two different mycotoxins are present in the same feed (Table 1). The issue gets more complicated when three or more mycotoxins are present together. Table 1 demonstrates that all the mycotoxins studied contributed to the toxicity of the feed, some additively and some synergistically, making the feed far more toxic than predicted. This paper proposes a methodology for evaluating the potential toxicity of a feed contaminated with multiple mycotoxins based on the published LD_{50} values for mycotoxins.

MATERIALS AND METHODS

LD_{50} values for various mycotoxins were compiled from the scientific literature for the calculation of total toxicity (Leeson et al., 1995; Table 2). For the purpose of explaining the total or potential toxicity, a poultry feed sample contaminated with 50ppb aflatoxin B_1, 50ppb T-2 toxin, 40ppb HT-2 toxin, and 2000ppb DON was considered (Table 3).

RESULTS

In our study, the poultry feed DON was the least toxic mycotoxin based on LD_{50} (Table 2). The TI for DON, therefore, was considered as one. The TI for aflatoxin B_1 was calculated by dividing LD_{50} value of the least toxic mycotoxin (DON in this case) by LD50 value of aflatoxin B_1 (Table 3). The same was repeated for T-2 toxin and HT-2 toxin. TI provides information on how many times a particular mycotoxin is more toxic than the least toxic mycotoxin.

When TI for a specific mycotoxin is multiplied by its concentration in the feed the resulting value represents the concentration of that specific mycotoxin expressed in terms of least toxic mycotoxin. In this way the concentrations of all the mycotoxins present in the feed can be standardized and summed to obtain a total concentration of least toxin mycotoxin.

In the example discussed above, the total mycotoxin toxicity to birds is expressed in terms of DON concentrations. Using the published information on mycotoxin limits, an estimate of whether 4793ppb DON is toxic to poultry or not can be made. The authors understand that mycotoxins will not induce mortality at all times and therefore, the use of LD_{50} values to define toxicity does have limitations. Since precise LD_{50} data is available, for most of the important mycotoxins in one-day-old chicks, this method provides a tool of estimating potential toxicity while considering mycotoxin interactions. This approach can be used until a more accurate method of defining total mycotoxin toxicity is available.

CONCLUSIONS

The presence of multiple mycotoxins in poultry feed is a common phenomenon. Such presence poses challenges in predicting potential toxicity of contaminated feeds to animals. Using the method described, a TI can be developed for each mycotoxin present in the feed based on their LD_{50} values. Combining the information on such indices along with their concentrations in the feed, all the mycotoxin concentrations can be expressed in terms of the least toxic mycotoxin. This approach may help to better predict multiple mycotoxin toxicity in the field.

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Table 1. Scientifically proven mycotoxin interactions in poultry.

Mycotoxins	Type of interaction
Aflatoxin B_1 X Ochratoxin A	Synergistic
Aflatoxin B_1 X Diacetoxyscirpenol	Synergistic
Aflatoxin B_1 X T-2 toxin	Synergistic
Aflatoxin B_1 X Cyclopiazonic acid	Additive
Aflatoxin B_1 X Deoxynivalenol	Additive
Ochratoxin A X T-2 toxin	Additive
Ochratoxin A X Cyclopiazonic acid	Additive
T-2 toxin X Deoxynivalenol	Synergistic
T-2 toxin X Fumonisin B_1	Additive
Fumonisin B_1 X Moniliformin	Additive
Fumonisin B_1 X Fusaric acid	Synergistic

Source: Devegowda and Krishnamurthy, 2005

Table 2. LD_{50} values for mycotoxins in one-day-old broiler chicks.

Mycotoxin	LD_{50} (mg/kg)
T-2 toxin	4.97
HT-2 toxin	7.22
Neosolaniol	24.87
Diacetoxyscirpenol (DAS)	3.82
Deoxynivalenol (DON)	140
Ochratoxin A	2.14
Citrinin	95
Aflatoxin B_1	11.50
Moniliformin	5.40

Source: Leeson et al., 1995

Table 3. Calculation of total poultry toxicity based on LD_{50} values for mycotoxins.

Mycotoxin	Concentration in feed, ppb	*Toxicity Index	**Mycotoxin equivalence, ppb
Deoxynivalenol (DON)	2000	1	2000
Aflatoxin B_1	50	12.17	609
T-2 toxin	50	28.17	1408
HT-2 toxin	40	19.39	776
	Total relative DON Concentration, ppb		4793

*LD50 value of the least toxic mycotoxin divided by LD_{50} value of the mycotoxin in question
**Concentration in feed (ppb) multiplied by Toxicity Index

AN EMERGING DISEASE IN MISSISSIPPI? A RETROSPECTIVE LOOK AT *PASTEURELLA MULTOCIDA* AND *MANNHEIMIA HAEMOLYTICA* ISOLATES FROM CHICKENS

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SUMMARY

A five year retrospective study (November 2006-December 2011) was conducted to determine the incidence of *Pasteurella multocida* and *Mannheimia haemolytica* and their antibiograms from chickens submitted to the Poultry Research and Diagnostic Laboratory. The prevalence of *M. haemolytica* increased over the last five years in both broiler and broiler breeder type chickens. *M. haemolytica* showed nearly complete resistance to novobiocin, tylosin, lincosamide, and tetracycline antimicrobials. *M. haemolytica* showed moderate to high sensitivity to sulfonamides, fluoroquinolones, and florfenicol. There was intermediate sensitivity for spectinomycin and erythromycin, and variable resistance to beta-lactam and aminoglycoside antimicrobials. In sharp contrast, *P. multocida* showed moderate to high sensitivity to beta-lactam, novobiocin, and tetracycline antimicrobials, but had similar antibiograms for the other antimicrobials. Sensitivities were determined using minimum inhibitory concentration (MIC). This study examines the trends over time of the number of isolates of these two organisms and their sensitivities, and suggests that an antibiogram should be established to optimize treatment.

An emerging disease? This study became of interest primarily due to an apparent rise in the number of isolates of *M. haemolytica* in our poultry industry over the last five years. Once classified as a *Pasteurella*, this organism has not typically been perceived as a pathogen in commercial poultry, at least not in the poultry industry in the southern United States. Interest was sparked when this organism started to show up in pure culture from "problem" broiler and breeder flocks. It was isolated from joints, wattles, lungs, abdomens, hearts, and other viscera. In breeder flocks, *M. haemolytica* appears to present very similar to what we typically see with *P. multocida* (fowl cholera) systemic infections (nagging mortality, decreased production, swollen wattles, peritonitis, and airsacculitis). Antiabong *et al.* (1) reported that *M. haemolytica* caused gross pathology, similar to that found with fowl cholera, in clinically ill and dead laying chickens in Nigeria.

MATERIALS AND METHODS

Samples were routinely submitted to the Poultry Research and Diagnostic Laboratory for microbiological culture and sensitivities. Samples (approximately 300 cases or swabs) were initially inoculated onto 5% sheep blood plates, Columbia CNA with 5% sheep blood plates and MacConkey agar plates. Plates were then incubated for 18 to 24 h at 37^0C. Suspect bacterial colonies were selected and identified by analysis with the Sensititre ARIS 2X system (15) utilizing the Sensititre GNID panel. Antibiotic sensitivities were determined by MIC with the Sensititre AVIAN1F panel following the suggested manufacturer protocols. Sensitivities were determined for the following antibiotics: beta-lactams (amoxicillin, ceftiofur, and penicillin), lincosamide (clindamycin), tetracyclines (doxycycline, minocycline, oxytetracycline, and tetracycline), fluoroquinolones (enrofloxacin), macrolides (erythromycin and tylosin tartrate), florfenicol, aminoglycosides (gentamicin, neomycin, and streptomycin), novobiocin, spectinomycin, and sulfonamides (sulfamethoxazole / trimethoprim, sulphadimethoxime, and sulphathiazole).

RESULTS

A total of 291 isolates of *M. haemolytica* (84) and *P. multocida* (207) were identified from November 2006 to December 2011 (Table 1). The overall prevalence of *M. haemolytica* gradually increased from n = 6 in 2006-2007 to n = 28 in 2010-2011. These isolates came from about as many broiler flocks (44 isolates) as broiler breeders (40 isolates). Both bird types show a similar increasing trend. For *P. multocida*, the prevalence increased from n = 33 in 2006-2007 to n = 56 in 2009-2010. The prevalence then decreased in 2010-2011 to n = 32. *P. multocida* isolates all came from broiler breeder type chickens.

M. haemolytica. *M. haemolytica* isolates were sensitive to ceftiofur (90% S), enrofloxacin (93% S), gentamicin (93% S), florfenicol (86% S), sulfamethoxazole/trimethoprim (83% S) and sulfathiazole (82% S). These results are similar to those

of Malik *et al*. (10) who reported *M. haemolytica* chicken isolates with sensitivities to ceftiofur (100% S), enrofloxacin (96-100% S), florfenicol (92-100% S), and sulfamethoxazole/trimethoprim (93-100% S). Similar findings have also been reported in other species. Berge *et al*. (3) reported 100% sensitivity to ceftiofur and florfenicol from isolates taken from sheep and goats. Diker *et al*. (6) reported high sensitivities to ceftiofur, enrofloxacin, gentamicin, and sulfamethoxazole/trimethoprim for sheep isolates. Priebe *et al*. (13) reported 100% sensitivity to florfenicol from pig isolates. Sensitivities in this study to ceftiofur, florfenicol, gentamicin and enrofloxacin are similar to findings previously reported in cattle (2, 7, 9, 11, 12, 16). Contrasting reports exist for sensitivities to sulfamethoxazole/trimethoprim in isolates from cattle that show variable to low sensitivities (7, 11).

There was intermediate sensitivity to spectinomycin in which over 89% of the isolates were moderately sensitive. Malik *et al*. (10) reported that only 0-50% of chicken isolates were sensitive to spectinomycin. In cattle, Schwarz *et al*. (14) reported that 98% of the isolates were sensitive, and Watts *et al*. (17) reported that 83% of the isolates were sensitive.

Isolates from this study revealed that there was resistance to novobiocin (100% R), tylosin (100% R), clindamycin (97% R), tetracycline antimicrobials (range of 80-90% R), and penicillin (70% R). Similar findings in chickens have been reported for penicillin (60-100% R) and tetracycline (72-100% R) by Malik *et al*. (10). Isolates from cattle have been reported to be resistant to penicillin, tylosin and tetracyclines (7, 9, 11, 12, 17). In contrast, Berge *et al*. (3) reported only a 5% resistance to tetracyclines in sheep and goat isolates.

Isolates showed mixed sensitivity results for amoxicillin (43% S, 21% I, 36% R), erythromycin (57% I, 43% R), neomycin (64% S, 22% I, 14% R), streptomycin (75% S, 4% I, 21% R), and sulphadimethoxime (43% S, 14% I, 43% R). Berge *et al*. (3) found that 100% of their sheep and goat isolates were sensitive to amoxicillin. Hendrikson *et al*. (7) found that most of their cattle isolates were also sensitive to amoxicillin. In contrast, Mevius *et al*. (11) found their cattle isolates to be resistant to amoxicillin. For erythromycin, Malik *et al*. (10) reported 0% of their chicken isolates were sensitive. Watts *et al*. (17) also reported a very low sensitivity of cattle isolates to erythromycin. In contrast, Post *et al*. (12) reported that cattle isolates showed moderate susceptibility to erythromycin and streptomycin. Finally, results similar to ours were reported regarding sulphadimethoxime sensitivity in chicken isolates (10) and in cattle isolates (12).

P. multocida. In sharp contrast to *M. haemolytica* isolates, *P. multocida* isolates showed moderate to high sensitivity to beta-lactam, novobiocin and tetracycline antimicrobials, but had similar antibiograms for the other antimicrobials. Our chicken isolates were sensitive to doxycycline (99% S), minocycline (99% S), sulfamethoxazole/trimethoprim (96% S), ceftiofur (95% S), florfenicol (94% S), amoxicillin and enrofloxacin (93% S), sulfathiazole (92% S), neomycin (89% S), oxytetracycline and tetracycline (86% S), novobiocin and sulphadimethoxime (85% S), and gentamicin (79% S). Similar findings have been reported in chickens by Huang *et al*. (8) for gentamicin, amoxicillin, ceftiofur, enrofloxacin, florfenicol, spectinomycin, tetracycline and sulfamethoxazole/trimethoprim.

There are also similar findings in other species. Berge *et al*. (3) reported a high sensitivity to amoxicillin (100% S), ceftiofur (100%), florfenicol (100% S), and to tetracyclines (95% S) in sheep and goats. High sensitivity to ceftiofur in cattle has been previously reported, as well (7, 11, 12, 16, 17). In addition, there are reports in cattle (7, 11, 16) and in swine (13) of high sensitivity to florfenicol. There have been mixed reports regarding sensitivity to gentamicin in cattle. Mevius *et al*. (11) reported a high sensitivity (84-98% S) whereas Post *et al*. (12) reported only a moderate sensitivity. Mevius *et al*. (11) also reported a high sensitivity (84-98% S) to neomycin, which is similar to our findings. There are also mixed reports for sensitivity to amoxicillin. In sheep and goats by Berge *et al*. (3) and in cattle by Hendriksen (7) there are reports of high sensitivity to amoxicillin whereas, Mevius *et al*. (11) reported variable results (47-84% S) in cattle. In contrast, Hendrikson reported a variable sensitivity (7) to enrofloxacin in cattle, but Mevius *et al*. (11) reported high sensitivity (84-98% S). Sensitivity to tetracycline similar to ours was reported in sheep and goats (3). Variable to high resistance to tetracycline has been reported in cattle (7, 11, 12, 17). Similar findings in cattle for sulfamethoxazole/trimethoprim were reported by Hendrikson *et al*. (7), but variable sensitivities were reported by Mevius *et al*. (11). There is a conflicting report by Post *et al*. (12) that reported a high resistance to sulphadimethoxime in cattle in contrast to the high sensitivity observed in our chicken isolates.

Similar to reports in cattle (12, 17), there was intermediate sensitivity to erythromycin (78% I) and spectinomycin (87% I). There are also conflicting reports for spectinomycin reported in chickens (8) and in cattle (11, 14) which showed high sensitivities (98% S, 84-98% S, 94% S respectively). There was high resistance (97% R) only to clindamycin and tylosin. The tylosin findings are similar to those reported in cattle by Post *et al*. (12). Isolates showed mixed

sensitivity results for penicillin (54% S, 30% I, 16% R) and streptomycin (22% S, 18% I, 60% R). Similar to our findings, Post *et al*. (12) reported only a moderate sensitivity to penicillin.

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Table 1.

	06-07	*07-08*	*08-09*	*09-10*	*10-11*	*Totals*
P. multocida	33	46	40	56	32	**207**
M. haemolytica	6	15	14	21	28	**84**
					Total	**291**

THE EFFECT OF A SEALANT ON PREVENTING LITTER BEETLE PENETRATION IN DIFFERENT TYPES OF INSULATION

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SUMMARY

Litter beetles, *Alphitobius diaperinus* (Panzer), are a known reservoir of several poultry pathogens. One area of the poultry house that beetles congregate is within the hard board insulation panels in the walls. Preventing beetles from entering the insulation will assist in controlling this problem since they will spend more time in contact with the insecticide which is typically applied to the litter. In this study we utilized eighteen 10 x 10 cm squares of three different insulation types. Half of the squares were sealed with ThermaCote, a high performance thermal and weather barrier that incorporates ceramic technology and is applied like a latex or acrylic paint. The remaining insulation squares were untreated (three reps/insulation type/treatment). The sealed and unsealed insulation squares were added to a litter beetle colony for seven wks. Inspections were performed weekly. After one wk there was significant damage to the untreated insulation that increasingly had gotten worse each wk; however, after seven wks there was no damage to properly coated insulation.

INTRODUCTION

Liter beetles are common pests in poultry and are well known for eating feed, disrupting chickens, harboring diseases, and causing damage to housing. They are nocturnal, cosmopolitan, have no natural enemies, and are attracted to ammonia. They have a well-defined life cycle, reaching maturity in 40 to 80 d under ideal conditions. These conditions are primarily temperature dependent with it being in the range of 21^{o}C to 35^{o}C. Litter beetles prefer an environment with about 12% moisture; however, they do thrive in moisture levels higher than this. Though moisture plays a role in litter beetle's life cycle the crucial factor for them to reproduce and mature is temperature.

Litter beetles have been associated with many diseases. The disease causing agent is carried either on the exterior of the beetle or inside the beetle's gut. Beetles pick up the disease causing agents by either crawling through an infected environment or by consuming an infected bird. Typically the disease-causing organism can be carried for two to three wks and for some agents longer. Diseases associated with beetles include IBD, Marek's, LT, RSS, *E. coli*, *Salmonella*, dermatitis, necrotic enteritis, aspergillosis, and coccidiosis (1).

In addition to directly affecting the birds, beetles do significant damage to housing. While they are searching for food and a place to pupate it is common for them to damage wood and insulation. The amount of damage is dependent upon the severity of infestation and the type of construction material. Even a fairly mild infestation can cause hundreds of dollars of repair to a house annually. This is the visible cost; this figure doesn't take into consideration the hidden cost of reduced house efficiency because of a reduction in the insulations R-value.

ThermaCote was originally developed to boost the performance of insulation in buildings by reducing or eliminating moisture, thermal transfer, thermal bridging, and conduction. It is a spray latex/acrylic material that incorporates ceramic technology. It can be applied to the exterior of the roof to act as a thermal shield and extend the life of the roofing material. Due to its ease of application and hard, impervious quality it was deemed a possible candidate to prevent litter beetle damage if applied to insulation board typically used in commercial poultry houses.

MATERIAL AND METHODS

For this study, approximately 1000 beetles were harvested from the Auburn University litter beetle colony. These beetles were placed into a 30-gallon plastic storage container, which contained 5 kg of chicken litter. To this container old chicken feed and a container of water were added. Throughout the course of this trial feed and water were maintained *ad libitum* for the beetles.

Three types of insulation boards were utilized in this study: expanded polystyrene foam (EPS), extruded polystyrene foam (XPS), and polyisocyanurate (Polyiso). For each of the three insulation board types, six 10 x 10 cm squares were cut out. For each insulation type, three of the six squares were treated with ThermaCote according to the manufacturer's specifications (2), while the other three were left untreated. All 18 squares were then added to the plastic container described above. Observations were taken each wk for seven wks.

RESULTS AND DISCUSSION

After one wk all of the untreated insulation boards had noticeable beetle penetration boreholes. The number of boreholes increased on the untreated insulation boards as the study progressed. The number of beetle penetration boreholes was dependent on the insulation type with EPS having the most holes, followed by XPS, and finally Polysio. Beetle penetration numbers correlate with the insulation board density, which directly correlates to the insulation value (R- value) of the board. Since EPS has the lowest insulation value (R value = 3.5) and is the least dense, it had the greatest amount of beetle boreholes. XPS and Polysio have higher R values (5 and 6.5, respectively) and had fewer beetle boreholes than EPS. By the end of the trial all of the untreated boards had similar numbers of beetle penetrations, regardless of insulation board type.

The insulation boards treated with ThermaCote had no litter beetle boreholes. The exceptions were those boards that were not fully coated with the product; however, even in those the number of boreholes was low, since the majority of the board was still covered.

In conclusion, litter beetles quickly damaged untreated insulation boards. The amount of damage over the first few wks seemed to be dependent upon the type of insulation board, with the less dense insulation boards having more beetle damage. Insulation boards properly treated with ThermaCote prevented litter beetles from boring into them. Further research needs to be performed to determine if other coating material will inhibit litter beetles boring into insulation board. Research needs to be performed to determine if ThermaCote can effectively protect a commercial poultry house from litter beetle damage.

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ADVANTAGES OF ELISA TEST OVER HI TEST TO EVALUATE A NEWCASTLE DISEASE VACCINATION PROGRAM

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INTRODUCTION

Newcastle disease (ND) is a lethal and highly contagious disease of poultry (1). It is enzootic in several countries in Africa, Asia, and South America. Vaccination using live and killed vaccines is a common practice in areas where the virulent strains are endemic in the commercial poultry (2); however, NDV is still responsible for direct and indirect economic losses. Outbreaks of ND in birds older than four wk old can be seen in two forms. The first form, respiratory distress, is common in well protected flocks, with low mortality. The second form is digestive or nervous symptoms (1-2%). The diagnosis can be difficult because of the two forms. The classic form of ND is seen in flocks with non-uniform immunity which results in common nervous and digestive signs as well as a drop in egg production and mortality rate higher than 5%. Repeated vaccination is required to protect chickens throughout life because immunity induced by the vaccination program lasts for only eight to ten wk. The frequency of re-vaccination largely depends on the risk of exposure and virulence of the field virus. In practice, vaccination of poultry against NDV does not always prevent periodic ND outbreaks (3).

The availability of standard sensitive serological test could facilitate diagnosis and accurate monitoring of vaccination programs. Hemagglutination Inhibition (HI) and ELISA are the most used serologic techniques for the detection of anti-NDV antibodies in chickens.

The aim of this study was to measure the ELISA and HI antibody response and productive performance of vaccinated White Leghorn type laying hens challenged with the Mexican Chimalhuacan ND strain at 25 wk old.

MATERIALS AND METHODS

Three groups of commercial leghorn laying hens, identified as A, B, and C, were used. All of them were raised in commercial farms, the vaccination program included live LaSota strain at one, three, five, six, and 15 wk of age, and killed vaccines at five, ten, and 15 wk. Group A received no killed vaccine at 15 wks old. Groups B and C received a different killed strain at five, ten, and 15 wk old (B= P2005, C= Uster). At 18 wk old, the hens were housed in three isolation rooms at the Departamento de Medicina y Zootecnia de Aves (DMZA), FMVZ – UNAM with 25 caged hens per group in each room. All birds received free access to fresh water. Feed and light programs were established according to standard management procedures.

Serum samples were taken at challenge, two, and four wk post challenge. All samples were tested by ELISA test (AffiniTech, LTD, Arkansas, USA), according to the kit insert, and HI test, according to the NOM-013-ZOO-1994 (4).

All hens were challenged at 25 wk old with the Mexican virulent standard challenge strain Chimalhuacan, by the ocular route with 10^6 $EID_{50\%}$ / 0.2 mL. After challenge, all groups were observed for four wk. Samples for virus isolation were taken from three hens at two and four wk post challenge. All surviving birds were humanely sacrificed at the end of the experiment. The necropsy was performed on ten hens per group and samples were taken for virus isolation. Clinical signs, egg production, and weight and external quality of the egg were recorded during the four wk post challenge. Protection against challenge was confirmed when birds did not show clinical signs, death, or reduction in egg quantity or quality (shell-less eggs).

ELISA and HI antibody titers, mortality, percent of egg production, and poor quality eggs were compared by ANOVA, and Chi square tests, respectively. Alpha was established as 0.05.

RESULTS

Effects of challenge are summarized in Table 1. Egg production was 96% for groups A and B, and 92% for group C at challenge. The mean egg production in the four wk after challenge period was lower ($P<0.05$) in group A than in groups B and C. Egg quality was better ($P<0.05$) in groups B and C when compared with group A. Mortality was higher ($P<0.05$) in group A than in groups B and C. There was not statistical difference in egg weight among groups. All hens from group A showed clinical signs more severe than those observed in the other groups.

The results of the ELISA and HI tests are shown in Table 1. The ELISA mean titer in group A was lower ($P<0.05$) than in groups B and C at challenge (25 wk old). There was no statistical difference in the ELISA titer among groups at two and four wk post-challenge. However, the ELISA titer in each group increased statistically ($P<0.05$) from challenge to 27 and 29 wk old. The HI geometric mean of the titer in group A was lower ($P<0.05$) than in groups B and C at challenge (25 wk old). There was no statistical difference in the HI titer among groups at two and four wk post-challenge. The HI titer increased statistically ($P<0.05$) from challenge to 27 wks\ old only in group A. Groups B and C showed increase in the HI titer two wk after challenge, but it cannot be considered as seroconversion since the increase was lower than fourfold the titer at challenge. HI titers remained almost the same from 27 to 29 wk old in all groups. The challenge virus was not isolated from any sample taken at two and four wk post challenge.

DISCUSSION

After challenge with the Chimalhuacan ND virulent strain, group A showed the most severe clinical signs, but a relative low mortality at 12%. It has been reported that HI titer between eight and 16 can prevent 90% of mortality, and with HI titer higher than 16, the mortality can be zero after challenge (5). This study proves that the HI MGT of 38 can protect the laying hens against the death, and that titer correlates with the 559 titer obtained with the ELISA kit used in the study (AffiniTech, LTD). However, this low antibody titer could not avoid the affect on egg production, which reinforces the need of having a high antibody titer before the onset of egg production. The vaccination program in group B resulted in 1,702 HI titer and 11,457 ELISA titer at challenge. It protected 96% of the hens against mortality, but allowed 18% reduction in egg production in the four wk period. The vaccination program in group C fully protected against mortality and allowed less than 10% reduction in egg production in the same period. Vaccination programs B and C drastically reduced the laid of shell-less eggs. These results agree with those who stated that HI titer higher than 128 can protect against mortality and egg quality alteration (5, 6).

The Chimalhuacan strain is the Mexican standard challenge virus, and apparently did not affect the egg weight, even when almost 26% of the laid eggs in group A were shell-less. A ND vaccination program for White Leghorn laying hens should be based on the degree of immunity necessary, the costs involved, and the exposure to local field viruses.

Hemagglutination inhibition test is the most widely used for measurement of antibodies against NDV since it is simple to perform; however, it is difficult to standardize among laboratories.

Seroconversion can be detected by HI only when there is a fourfold increase of antibody titer in suspicious flocks, besides, the laboratories commonly run the test to get titers as high as 256 or 4096. In this study, only group A showed seroconversion two wk after challenge, but groups B and C did not meet the criteria of increasing four times the titer. The ELISA test was useful to detect the infection by seroconversion in flocks with high humoral immunity against ND (groups B and C).

The ELISA technique is widely accepted within poultry industry as the most reliable serological monitoring tool. The software provided by the manufacturers has the ability to present individual or grouped flock titer information in both graphic and numerical formats, which in turn facilitates the data analysis. Other advantages of ELISA include its sensitivity, specificity, quick processing time of multiple samples, its objectivity, reproducibility, and computer generated versatile database. The additional advantage of ELISA over HI is that, while screening for NDV antibodies, the occurrence of non-specific antihemagglutinin and inhibitors is not important. The HI test and ELISA may measure antibodies to different antigens. Depending on the system used, ELISAs may detect antibodies to more than one antigen while the HI test is probably restricted to those directed against the HN protein. Comparative studies have demonstrated that the ELISAs are reproducible and have high sensitivity and specificity and they have been found to correlate well with the HI test (Adair *et al.*, 1989).

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Table 1. Effects of challenge with Chimalhuacan strain of NDV on White Leghorn type laying hens.

Group	Production at challenge (%)	Production after challenge (%)[1]	Shell-less eggs	Mortality (%)	Egg weight (g)	Clinical signs
A	96	28.18a	25.75a	12a	53.3	Anorexia, ruffled feathers, severe depression
B	96	78.04b	2.6b	4b	54.7	Scarce green diarrhea
C	92	82.72b	1.52b	0b	54.1	Scarce green diarrhea

1 Different letter in the same column denotes statistical difference, ($P<0.05$).

Table 2. Antibody titers measured by ELISA and HI in White Leghorn type laying hens.

	ELISA			HI		
	Age (wk)[1]			Age (wk)		
Group	25	27	29	25	27	29
A	559a/a	24624a/b	30338a/c	38a/a	4096a/b	4096a/b
B	11457b/a	24929a/b	30283a/c	1702b/a	4096a/a	3566a/a
C	18132b/a	23879a/b	30467a/c	2464b/a	4096a/a	4096a/a

1Different letter at the left of the slash denotes statistical difference in the values of the same column. Different letter at the right of the slash indicates statistical difference in the values of the same row and serological test, ($P<0.05$).

THE USE OF pET SUMO PROTEIN EXPRESSION SYSTEM FOR MAXIMAL PRODUCTION OF APOPTIN WITHIN *E. COLI* CELLS

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SUMMARY

Apoptin, a non-structural protein of chicken anemia virus, drives apoptosis in chicken thymocytes and chicken lymphoblastoid cell lines. It has been shown in previous studies that apoptin is also required for viral replication (1). To further study the hypothesis of how apoptin is involved, a protein expression system is needed. Champion™ pET SUMO Expression System is utilized to produce the highest quantity and quality of apoptin. VP3, the gene transcript of apoptin, is cloned into a Champion pET SUMO plasmid. DNA sequencing is used to determine whether the clone was inserted in the right orientation. Plasmids are added to the competent *E. coli* cells for protein expression and the product is run on a SDS-PAGE for protein size analysis (~21kDa). Western blot using mono-specific antibodies identify the product as apoptin. Therefore, Champion pET SUMO Expression System is suitable for large scale production of apoptin required in the future study.

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EFFECT OF AUDITS AND CAMERAS ON BIOSECURITY COMPLIANCE – RESULTS SIX MONTHS LATER

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INTRODUCTION

Constant application of biosecurity measures is essential for the success of all types of animal productions. Despite its importance, there is a paucity of studies that have investigated strategies to enhance biosecurity compliance at the farm level. The main objective of this study was to determine the impact of audits and visible cameras on short term (first two wk) and medium term (six months later) compliance with biosecurity measures when entering and exiting poultry barns. Short term results were presented at the last Western Poultry Disease Conference meeting in Sacramento .

SUMMARY

Twenty-four commercial farms were selected. The inclusion criteria were having at least one farm employee and a biosecurity protocol in place for entering and exiting barns. Production types targeted were meat-type farms (chickens or turkeys), commercial layers, and breeder farms. One barn was selected for each farm. Farms were systematically assigned into three groups (control, audit, and camera) of eight farms. For the control group, there was no intervention, other than installing a laminated poster with the grower's list of required measures. A hidden camera was installed in the barn entrance. For the audit group, there was also a hidden camera in the entrance

of the barn, as well as a poster detailing the required biosecurity measures. An additional poster highlighted the farm's participation in a biosecurity audit project. There were three none announced audits over a six-month period and they consisted in evaluating the availability and feasibility of biosecurity measures. Biosecurity principles were discussed with all employees during each audit and changes in the barn entrance setup were made to facilitate daily compliance. For the camera group, in addition to the poster listing the required measures, there was a visible camera in the entrance of the barn for six months and a poster advising that a constant monitoring was done. For ethical reasons, all employees and visitors had to be informed that they may be filmed at some point and they had the opportunity to refuse participating.

A total of 2748 visits featuring 259 individuals were included in the study. There were 136 employees and 123 visitors. The presence of a visible camera in a barn entrance enhanced short term compliance. However, six months after its installation, compliance with area during the visit (respecting contaminated and clean areas) was the only biosecurity measure that was enhanced at the medium term assessment. Compliance declined over time in all study groups, even within the control group suggesting that the presence of a new poster with the grower's list of required biosecurity measures probably enhanced short term compliance. Bimonthly audits did not have any impact on medium term compliance. Although audits seemed ineffective in our study, we must keep in mind that the auditor in this case, was not in a position of authority over the employees.

Farm factors. Having more than five barns on the site was positively associated with boot compliance. This may be related to concerns due to the risk of disease transmission between barns. Employees of larger farms may be more concerned with heavier traffic, may be more used to frequent boot changing, or may have received more training. Also, having a physical barrier and sufficient space in barn entrances were also positively associated with area compliance. Infrastructures should facilitate or even encourage people to comply. Barn entrance design should permit a logical and practical application of biosecurity measures on a daily basis. Replacing area delimitation lines with physical barriers and providing sufficient space to perform required biosecurity protocol should enhance and contribute to maintain compliance.

Individual factors. Compliance with areas at entrance was better for men than women. This may be related to experience and training, but may also be a statistical finding, since men did not perform significantly better for any of the other required measures. Compliance with areas at exit was better when plastic boots were used instead of farm boots. It is probable that respecting areas was enhanced when plastic boots were used because they are easier to remove. However, plastic boots are not durable and should not be reused.

Visit factors. The duration of the visit was strongly related to boot, coveralls, and hand washing compliance and to overall visit compliance. Since the risk of disease transmission may not be proportional to the time spent working in a barn, it is important to be aware of people coming in for short visits because they may be less likely to comply. Locking doors is needed to avoid unexpected visits when, for example, a visitor is looking for the grower. Boot and coveralls compliance were better in the morning compared to the afternoon. It may be due to more demanding farm activities performed in the morning, such as picking up dead birds. It is not justified to alter biosecurity measures depending on the time of day. The presence of the grower enhanced hand washing compliance, but reduced compliance with coveralls. Washing hands may be perceived easier and faster than donning coveralls. Growers' attitudes and leadership were not evaluated, but may be related to their employees' compliance. Finally, family members were less likely to comply with the required biosecurity protocol.

Continuous training programs should address issues related to visits by all poultry personnel and visitors. Of particular importance is emphasizing that biosecurity measures must be applied with the same rigor independently of the duration and the moment of the visit. Improving barn entrance design by replacing area delimitation lines with physical barriers and facilitating the application of each measure by providing enough space and adequately positioned equipment should contribute to enhancing and maintaining compliance. Since the presence of an observer may affect compliance negatively, it is also important to train employees to enforce compliance when more than one person enters or leaves a barn.

Finally, we must stress that no single intervention or corrective action can, in itself, solve the problem of low biosecurity compliance.

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Table 1. Odds ratio estimates (95% confidence intervals) from multilevel logistic regression models describing compliance for each biosecurity measure required on Québec poultry farms six months after initial intervention (1).

Variables		Biosecurity measures (Odds ratios and 95% confidence intervals)					
		Boots	**Areas at entrance**	**Areas during visit**	**Areas at exit**	**Coveralls**	**Hands at entrance**
Comparison of study groups at the medium term assessment	**Audits** (ref: control group)	0.72 (0.06-9.1)		0.11 (0.004-3.5)		0.43 (0.01-13.7)	12.4 (0.48-319.9)
	Camera (ref: control group)	3.6 (0.27-45.9)		**28.5** (1.8-440.1)		8.3 (0.64-106.7)	8.4 (0.66-107.7)
Comparison of assessment periods within each study group	**Control group at medium term** (ref: control group at short term)	0.72 (0.42-1.2)		0.35 (0.06-1.9)		**0.40** (0.20-0.79)	**0.11** (0.05-0.22)
	Audit group at medium term (ref: audit group at short term)	**0.37** (0.19-0.70)		**0.04** (0.01-0.35)		**0.09** (0.02-0.55)	0.93 (0.36-2.4)
	Camera group at medium term (ref: camera group at short term)	**0.02** (0.01-0.09)		**0.33** (0.16-0.68)		**0.40** (0.16-0.99)	**0.16** (0.09-0.31)
Easy to comply (ref: intermediate)			**14.1** (1.1-184.1)		**12.7** (2.0-82.2)		
Easy to comply (ref: difficult)			**22.9** (1.3-417.7)		**13.1** (1.8-98.1)		
Duration of visit (ref: < 5 min)	5-17 min	**7.5** (4.2-13.5)		**0.18** (0.05-0.63)		**2.3** (1.1-4.5)	**2.0** (1.1-3.8)
	17-54 min	**17.0** (8.4-34.1)		**0.09** (0.03-0.31)		**13.4** (5.1-34.9)	**3.0** (1.6-5.4)
	≥ 54 min	**17.1** (7.9-37.1)		**0.09** (0.02-0.33)		**14.0** (5.6-35.5)	1.8 (0.98-3.3)
Morning		**1.5** (1.0-2.1)				**3.7** (1.9-7.1)	
≥ 5 barns		**17.7** (2.0-153.5)					
Plastic boots (ref: farm boots)					**5.0** (1.6-15.8)		
Men			**6.3** (1.4-27.5)				
Presence of the grower						**0.15** (0.05-0.43)	**3.1** (1.2-8.3)
Grower's family				**0.06** (0.01-0.51)			

PRESENTATION OF AN INTERACTIVE BIOSECURITY TRAINING TOOL

M. Racicot, J.-P. Vaillancourt, and G. Lambert

INTRODUCTION

The effectiveness of biosecurity measures depends largely on the consistency of their applications by all those involved in poultry production. Unfortunately, poor biosecurity compliance has been reported repeatedly in poultry, as well in all other major animal productions. A lack of knowledge or understanding of biosecurity principles among farm employees and visitors has already been proposed in several studies to explain low or variable compliance (1,2,4). There seems to be a need to improve understanding of biosecurity measures by demonstrating why and how to apply them.

SUMMARY

A new multimedia interactive training tool was developed based on recent results on biosecurity compliance (3). It is a CD that features a complete review of the biosecurity literature with 76 articles on barn entrance and exit, and more particularly on boots, coveralls and hand hygiene, hairnet, footbath, logbook, entrance sanitation, carcass disposal, and area delimitation. Three videos also explain how to enter and exit from a barn when area delimitation is a bench or a red line and when the person is an employee or a visitor. The interactive portion of the CD is comprised of 29 questions. There is a series of 22 questions, all including video clips, designed to highlight common biosecurity mistakes. They offer to view a video followed by a multiple choice question. An instant feedback is provided for any selection made by the user. Seven other questions feature videos where the user must click on them at the moment a biosecurity breach is observed. An instant feedback is also offered for this series. Finally, there is a section where the user has to design a barn entrance with three zones (contaminated, intermediate, and clean; this is based on the Danish entrance design recommended in many commercial swine operations. We believe that this design is superior to the traditional two zone design found on many swine and poultry farms. It facilitates compliance in the application of biosecurity measures). The exercise is to move pictures and actions in the right zone. This training tool may be used by individuals or as part of small group training sessions. In the context of assisted training sessions, the trainer can instruct participants to consider the intermediate zone as the line separating two zones (clean and contaminated). The current training tool was originally designed in French, but will also be produced (and available at no cost) in English in the near future.

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2001-2011 AVIAN BACKYARD DISEASES FREQUENCY IN CALIFORNIA

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SUMMARY

Electronic information from avian backyard submissions to the California Animal Health and Food Safety system between January 2001 and November 2011 was analyzed for determining disease frequency by etiology in California. A total of 3,372 submissions were considered. The vast majority of the submissions were chickens, but also included turkeys, waterfowl, pigeons, and game birds.

Bacterial diseases. Diseases caused by bacterial infections were the most common etiologic diagnoses with a total of 598 cases. Their frequency was as follows:

Escherichia coli infection, 95
Mycoplasma gallisepticum infection, 58
Mycoplasma synoviae infection, 32
Fowl cholera, 23
Staphylococcus aureus infection, 14
Clostridium perfringens infection, 13
Botulism, 13
Avibacterium paragallinarum infection, 12
Salmonella Enteritidis infection, 6
Pseudomonas aeruginosa infection, 4
Other bacterial infections with 3 or less cases, 16
Bacteria not identified, 170

Viral diseases. Diseases caused by viral infections were the second most common etiologic diagnoses with a total of 523 cases. Their frequency was as follows:

Marek´s disease, 321
Avian leucosis, 60
Avian pox, 51
Infectious laryngotracheitis, 23
Avian paramyxovirus 1 infection, 12
Duck viral enteritis, 5
Infectious bursal disease, 4
Avian encephalomyelitis, 3
Very virulent infectious bursal disease, 3
Other viral infections with two or less cases, 6
Virus not identified, 12

Parasitic diseases. Diseases caused by parasites were the third most common etiologic diagnoses with a total of 449 cases. Their frequency was as follows:

Coccidiosis, 122
Ascaridiasis, 70
Cestode infection, 56
Histomoniasis, 25
Trichomoniasis, 24
Louse infection, 17
Scaly legs, 15
Capillariasis, 14
Heterakis sp. infection, 14
Nematode infection, 13
Northern fowl mite infection, 8
Flea infection, 6
Schistosoma sp. infection, 4
Tetrameres sp. Infection, 3
Other parasitic infections with 2 or less cases, 13
Parasite not identified, 32

Management related and miscellaneous diseases. Management factors and miscellaneous diseases occupied the fourth place of etiologic diagnoses with a total of 233 cases. Their frequency was as follows:

Yolk peritonitis, 91
Trauma, 71
Foreign body, 23
Predator attack, 10
Obesity, 7
Cannibalism, 7
Dehydration, 7
Heart failure, 4
Injectable product reaction, 3
Starvation, 3
Heat stress, 2
Obstruction, 1

Neoplastic diseases. Neoplastic diseases ranked fifth of etiologic diagnoses with a total of 231 cases. Their frequency was as follows:

Adenocarcinoma, 108
Reproductive neoplasia, 35
Carcinoma, 12
Carcinomatosis, 11
Lymphoma, 9
Hemangioma, 3
Lymphosarcoma, 3
Leiomyosarcoma, 3
Pancreatic carcinoma, 2
Squamous cell carcinoma, 2
Other neoplasia cases with one occurrence, 13.
Neoplasia cases with no further description, 4

Metabolic diseases. Metabolic disorders accounted for the sixth place of etiologic diagnoses with a total of 139 cases. Their frequency was as follows:

Fatty liver syndrome, 46
Amyloidosis, 11
Hypoxia, 1
Metabolic cases with no further data, 81

Fungal diseases. Diseases caused by fungal infection ranked seventh in etiology for disease diagnoses with 109 cases. Their frequency was as follows:

Aspergillosis, 49
Candidiasis, 26
Crop mycosis, 14
Penicilium spp. infection, 2
Zygomycetes spp. infection, 2
Dermatophite infection, 2
Trichophyton spp., 1
Mycotic cases with no further identification, 13

Toxic diseases. Toxicants ranked eighth in etiology for disease diagnoses with 69 cases. Their frequency was as follows:

Zinc, 12
Lead, 10
Anticoagulant, 10
Silica, 8
Aspiration, 7
Sodium, 6
Oxalates, 2
Vitamin A, 2
Other toxicity cases with one occurrence, 10.
Toxicants no further data, 2

Nutritional diseases. Diseases due to nutritional deficiencies occupied the last place of etiology for diagnoses with 56 cases. Their frequency was as follows:

Rickets, 18
Vitamin A, 11
Vitamina E, 5
Copper, 3
Iodine, 2
Riboflavin, 1
Manganese, 1
Zinc, 1
Nutritional no further data, 14

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

CONTROLLING BROILER LITTER AMMONIA EMISSIONS WITH CHEMICAL LITTER AMENDMENTS

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SUMMARY

In commercial broiler houses, large quantities of ammonia are volatilized from the litter. This ammonia can cause burns and lesions to mucosal tissue around the eyes, trachea, and lungs. The damage will not only affect weight gain and performance, but add extra stress on the birds which may lead to infections. Reducing litter ammonia is commonly achieved by the use of various chemical litter amendments or acidifiers. These amendments lower litter pH and trap the ammonia before it is volatilized. In this study several acidifying litter amendments were chosen. These litter amendments included alum, sodium bisulfate, and sulfuric acid. Amendments were applied to litter at three application rates. Alum and sodium bisulfate were applied at 22.7, 45.4, and 60 kg/92.9 m^2 and sulfuric acid was applied at 9.07, 18.14, or 27.21 L/92.9 m^2. Ammonia measurements were taken one h before treatment and then every 24 h up to 96 post treatment.

Results of the study showed significant decrease in ammonia levels after 24 h regardless of amendment type, or application rate ($P<0.05$). Results also show significant decrease in ammonia levels between untreated control and treated litter, regardless of treatment type or application rate ($P<0.05$). This decrease in ammonia concentration can be attributed to a lowering of litter pH that occurred after treatment ($P<0.05$). Initial observations showed that the average litter pH was eight or greater. After treatment with acidifiers, litter pH was reduced, with the lowest pH being achieved by sulfuric acid at 2.1 after 24 h.

The observed reduction in litter ammonia volatilization was expected. Finding insignificant differences between recommended application rates and lower application rates was unexpected. These differences were noticed for the duration of the experiment; however, after an extended amount of time the lower application rate would not be as effective as higher rates. These chemical amendments, regardless of application rate, were shown to be an effective means of ammonia volatilization and therefore beneficial to broiler type chicken health.

(This work will be published in *Poultry Science*.)