PROCEEDINGS OF THE SIXTIETH WESTERN POULTRY DISEASE CONFERENCE

March 20-23, 2011 Sacramento, CA

SPECIAL ACKNOWLEDGMENTS

The 60th Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and to help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. More than 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 Poultry 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.**, **Intervet/ Schering-Plough Animal Health** and **Merial Inc.** 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.

Dr. Larry Allen, Program Chair for the 60th WPDC, would especially like to thank all those who submitted titles for their contributions to the success of this year's conference. Without their substantial efforts the Conference could never come to fruition. I extend sincere thanks also to all the presenters while we worked together to organize the program. Some presenters originally requested poster presentations and were willing to change to an oral presentation of their paper, or vice versa. This flexibility on the presenters' part helped keep us within time and space constraints of the program and the poster viewing room. Many thanks to my colleagues on the WPDC Executive Committee for their kind assistance and advice during the preparation of this program. Thanks also to the moderators who graciously accepted the opportunity to serve the Conference and keep the sessions orderly and on time. Finally, I gratefully acknowledge the assistance of the Staff at UCD Conference Events and Visitors Services for registering and assisting our guests and making the conference come together. Thank you again, one and all who made this milestone 60th WPDC a reality.

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

We thank Dr. David Frame for editing and producing another outstanding Proceedings of this meeting. Dr. Frame is indebted to Ms. Sherry Nielson for her tireless dedication in proofreading and formatting the Proceedings for publication. We express our gratitude to all authors who submitted manuscripts. A special thanks goes to **Pfizer Poultry Health** for their monetary sponsoring of the flash drives for this year's meeting. We again acknowledge and thank **Ominpress** (Madison, WI) for the handling and printing of this year's Proceedings and for electronic reproduction of the meeting proceedings on flash drives. Once again, we acknowledge Bruce Patrick (Graphic Communications, Brigham Young University) for the cover design of the printed Proceedings.

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60th WPDC PROCEEDINGS

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Copies of these Proceedings are available from: Dr. R. P. Chin
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50th WPDC Anniversary CD-ROM. This CD contains all printed proceedings of the first fifty Western Poultry Disease Conference meetings. Copies can be purchased from the AAAP: E-mail: aaap@aaap.info. Web: http://www.aaap.info.

Five-year Compilation (2002–2006) Proceedings of the WPDC. This CD contains the printed proceedings of the 51st through the 55th Western Poultry Disease Conferences. Copies can be purchased from the WPDC Secretary-Treasurer.

MINUTES OF THE 59TH WPDC ANNUAL BUSINESS MEETING

Secretary-Treasurer Chin called the meeting to order on Monday, 19th April 2010, at 5:00 PM, at the Fairmont Hotel Vancouver, Vancouver, BC, Canada. There were 24 people in attendance**.**

APPROVAL OF 58th WPDC BUSINESS MEETING MINUTES

The minutes from the 58th WPDC business meeting were reviewed and a motion was carried to approve them as printed in the Proceedings of the 59th WPDC.

ANNOUNCEMENTS

Dr. Chin acknowledged all the contributors; in particular, those contributing at the Benefactor level, which included the American Association of Avian Pathologists, Intervet/Schering-Plough Animal Health and Pfizer Poultry Health. He 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. Dr. Chin asked that we remember Dr. Charles Whiteman and Mr. Lloyd Merrill (Merrill's Poultry Farm, Paul, ID), who passed away the past year.

REPORT OF THE SECRETARY-TREASURER

Dr. R. Chin presented the Secretary-Treasurer report. First, he discussed the budget for the 57th WPDC in Mexico. Previously, he did not report all the expenses to ANECA for the budget. There was a net gain of $49,402.02 which ANECA agreed to distribute equally with WPDC. Hence, there was an overall gain of $24,701.01 for WPDC. Since ANECA raised more than three times the amount in sponsor/exhibitors contribution, the WPDC Executive Committee agreed to ask ANECA if, rather than give a lump payment to WPDC, that they would contribute at least US$1000 each year to WPDC. ANECA agreed to do so.

Dr. Chin then discussed the budget for the 58th WPDC. There were only 184 registrants – a 10% decrease from the expected 200 registrants. There was $24,050 in contributions with a total income of $59,525. Total expenses were $72,562.85, for a net loss of -$13,037.85. The current balance in the WPDC account was $61,600.85.

Contributions for this year's meeting (59th WPDC) were excellent at $34,650.00 and there is a higher registration of 219 at the beginning of the meeting. Unfortunately, this is lower than our previous meeting in Vancouver at which we had 256 registered. In addition, expenses were estimated to be considerably higher this year because of hotel costs and the weak currency exchange rate for USA. Dr. Chin estimated that there would be an overall loss of about $14,000 again this year.

The Executive Committee recommended an increase in registration fees. A discussion as to how much to raise the fees ensued. A motion was made and approved to raise the registration fees for presenters to $175 and for non-presenter, advance registration to $250. (Note: this does not include the ACPV workshop.)

The levels of contributions and their entitlements were discussed. It was agreed to leave the levels as is, but to change the complimentary registration entitlements. 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. In addition, sponsorship of the proceedings jump drive was discussed. It was suggested that a company should pay the entire cost of producing the jump drive if they want their logo on it.

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

OLD BUSINESS

None discussed.

NEW BUSINESS

In 2011, the 60th WPDC will be in Sacramento, CA. The Holiday Inn Capitol Plaza has been reserved for March 20-23, 2011.

Dr. Chin reported that the WPDC Executive Committee nominated Dr. Vern Christensen (retired, but recently moved to Utah) for Program Chair-elect of the 61st WPDC in 2012. There were no other nominations and Dr. Christensen was elected unanimously as program chair-elect. Dr. Chin nominated the following officers for 2010-2011:

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

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

Dr. Chin opened a discussion regarding venues for the 61st WPDC in Arizona. He received a number of proposals and asked Dr. Sharma, who recently moved to Arizona, to review them. Dr. Sharma visited a number of the venues and recommended we look at three in Scottsdale, AZ. They are the Scottsdale Resort & Conference Center, the DoubleTree Paradise Valley Resort, and the Radisson Fort McDowell Resort, in that order. There are pros and cons for each location. The group asked Dr. Chin to ask the Scottsdale Resort & Conference Center if there are any other dates available in March with the $149 guest room rate, and what is the food & beverage minimum. In addition, Dr. Chin was instructed to ask the DoubleTree if the guest room rate can be decreased. Dr. Cutler also said that he will be in Phoenix area the first part of May 2010 and can visit these venues.

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

The meeting was adjourned at 5:50 PM.

TABLE OF CONTENTS

Note: Both the oral and poster presentations of the 60th WPDC are listed below in alphabetical arrangement by presenter. Authors and titles of all papers scheduled for presentation at the conference are listed.

PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATED FROM RETAIL MEAT: NATIONAL ANTIMICROBIAL RESISTANCE MONITORING SYSTEM (NARMS) 2002-2008

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ABSTRACT

Retail meat samples (29,813), including chicken breast (n=7,457), ground turkey (n=7,419), ground beef (n=7,481) and pork chops (n=7,456) were collected during 2002-2008. A total of 2,203 *Salmonella* isolates were recovered and analyzed for antimicrobial susceptibility and genetic relatedness using PFGE. Among the isolates, 1,112 (50.5%) were from turkey, 909 (41.3%) from chicken, 98 (4.4%) from beef and 84 (3.8%) from pork. Sixty two serotypes were identified, and the top five serotypes were Heidelberg (20%), Typhimurium (11.8%), Kentucky (11.7%), Hadar (10%), and Saintpaul (8.3%). A total of 1,420 (64.5%) isolates displayed resistance to $\geq$1 of the 15 antimicrobials tested, and 224 (10.2%) were resistant to $\geq$5 antimicrobial classes. The most common resistance was to tetracycline (47.4%), followed by streptomycin (36.4%), ampicillin (27.8%), and sulfamethoxazole (27.6%); and to lesser extent, to gentamicin (15.4%), kanamycin (13.9%), amoxicillin-clavulanic acid (13.1%), ceftriaxone (12%), ceftiofur (11.8%), cefoxitin (11.7%), chloramphenicol (2.6%), nalidixic acid (1.4%), and trimethprim-suflamethoxazole (0.8%). All isolates were susceptible to amikacin and ciprofloxacin. PFGE analysis revealed a genetically diverse population; however, several clones were repeatedly recovered from different retail meats, brands and stores over the seven year period. Results indicate that multiple antimicrobial-resistant salmonellae were present in retail meats, and stress the need for surveillance of food borne pathogens in retail foods.

INTRODUCTION

Non-typhoid *Salmonella* is one the most important food borne pathogen. As with many bacterial pathogens, antimicrobial resistance in non-typhoidal *Salmonella* has become a major public health concern (1,2,3). The increased international attention to the risk of antibiotic use in animal production helped spur the development of numerous surveillance systems and networks (4). In the U.S., the National Antimicrobial Resistance Monitoring System (NARMS) was established in 1996 to monitor antimicrobial resistance in food borne pathogens, and identifies the source and magnitude of antimicrobial resistance in the food supply. The objective of this study was to determine the prevalence, antimicrobial susceptibility and genetic relatedness of *Salmonella* strains isolated from fresh retail meat purchased in the U.S. during 2002-2008.

MATERIALS AND METHODS

Bacterial isolates. A total of 2,203 *Salmonella* were recovered from 29,813 meat samples, including chicken breast (n=7,457), ground turkey (n=7,419), ground beef (n=7,481) and pork chops (n=7,456) collected during 2002-2008 from ten FoodNet sites. The detail information of sampling, isolation, identification and serotyping can be found at http://www.fda.gov/cvm/narms_pg.html.

Antimicrobial susceptibility testing. *Salmonella* isolates were assayed for susceptibility to 15 antimicrobials used by NARMS. Antimicrobial minimum inhibitory concentrations (MIC) of *Salmonella* isolates were determined via the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH) and interpreted according to CLSI/NCCLS standards where available. The following antimicrobials were tested: amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim/ sulfamethoxazole.

Pulsed-field gel electrophoresis (PFGE). Pulsed-field gel electrophoresis was performed according to the protocol developed by CDC (CDC; http://www.cdc.gov/pulsenet/protocols.htm).
S*almonella* were analyzed by *Xba*I and *Bln*I. PFGE results were analyzed using the BioNumerics Software (Applied-Maths, Kortrijk, Belgium).

RESULTS

Prevalence of *Salmonella* in the retail meats. Overall, 7.4% (N=2,203) of 29,813 retail meat samples were contaminated with *Salmonella*, with highest prevalence in ground turkey (14.7%) followed by chicken breast (11.8%). Both beef and pork has less than 2% of *Salmonella* contamination rate.

Among the isolates recovered, 62 serotypes were identified, and the top five serotypes were *S*. Heidelberg (n=441, 20%), Typhimurium (n=259, 11.8%, including Typhimurium var. Copenhagen and S.I 4, 5, 12: i :-), Kentucky (n=258, 11.7%), Hadar (n=220, 10%), and Saintpaul (n=182, 8.3 %).

Antimicrobial susceptibility profiles. The most common resistance was to tetracycline (47.4%), followed by streptomycin (36.4%), ampicillin (27.8%), and sulfamethoxazole (27.6%); and to lesser extent, to gentamicin (15.4%), kanamycin (13.9%), amoxicillin-clavulanic acid (13.1%), ceftriaxone (12%), ceftiofur (11.8%), cefoxitin (11.7%), chloramphenicol (2.6%), nalidixic acid (1.4%), and trimethprimsufla-methoxazole (0.8%). All isolates were susceptible to amikacin and ciprofloxacin.

Antimicrobial resistance differs in *Salmonella* by meat types and serotypes. For ground turkey isolates, the resistance has statistical significant increased over the years for Gen, Str, Amp, Amc and Tet, and decreased for Nal. For chicken breast isolates, the resistance was significantly increased for Tet and Fis. Chicken isolates remain the highest percentage of resistance to third generation of cephalosporins (Tio/Axo, 19.8%) compared to isolates from turkey meat (5.4%). Since there are low number of isolates from beef and pork, the resistance trends was not able to determine.

MDR *Salmonella* was isolated from all four types of meat, with higher number of MDR recovered from poultry meats. Overall, 31.8% and 10.2% *Salmonella* were resistance to $\geq$ 3 and $\geq$5 antimicrobial classes, respectively. MDR-AmpC phenotype, which showed resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline (ACSSuT), plus resistance to amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, and ceftiofur was seen in many *Salmonella* serovars, such as Dublin, Enteritidis, Heidelberg, I.4, 12: r:-, Kentucky, Newport, Saintpaul, and Typhimurium var. Copenhagen. MDR patterns varied within and between serotypes.

PFGE profile. PFGE fingerprinting profiles showed that *Salmonella*, in general, were genetically diverse. PFGE profiles showed good correlation with serotypes and in some instances, antimicrobial resistance profiles. Our PFGE data showed that some clones repeatedly contaminated the same meat product brand, in some instances being distributed among store chain outlets in the different states. Other clones appear to spread among different meat product brands and various store chains in the several states monitored. Many of MDR clones were repeated to detect from different states over the years. Many of PFGE profiles showed indistinguishing PFGE profile from human clinical isolates.

CONCLUSIONS

In summary, our results demonstrated a high prevalence of *Salmonella* recovered from retail poultry meat and many of *Salmonella* serotypes have been emerged to be MDR. Because retail meats represent a point of exposure close to the consumer, ongoing testing and monitoring the prevalence of antimicrobial resistance food borne pathogens from domestically produced meat commodities will help to better understand risks associated with antimicrobial resistance enteric pathogens in domestic retail meat supply, including the detection of new resistant genotypes should they arise. The integration of susceptibility data with PFGE analysis by the NARMS and PulseNet programs in the U.S. is important to understand emerging MDR food borne pathogens, and how MDR disseminates in the animal production environment, retail foods, and humans.

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PROPER USE OF THERAPEUTIC ANTIMICROBIALS IN POULTRY

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INTRODUCTION

Antibiotics are essential tools to maintain and restore poultry health when flocks are affected by pathogenic bacterial infections. Antibiotics have been available to poultry producers for disease prevention, treatment and control for five decades and continue to be irreplaceable tools for the prevention, treatment and control of bacterial diseases. For the same length of time antibiotics have also been added to poultry feeds to prevent disease, improve growth rate and feed efficiency. Although not recognized until the banning of the antibiotic growth promoters (AGPs) by the European Union (EU), the AGPs were preventing subclinical enteric disease and maintaining enteric health, which in turn resulted in better growth rates and feed conversions, this was most evident in Denmark where use of antibiotics for treatment of sick animals has increased each year following the ban of the AGPs in spite of the size of the food animal population remaining stable (1). However, increased concerns with the global development of multi-antibiotic-resistant infections in humans have brought much more scrutiny upon the way antibiotics are used in animal agriculture in general and poultry production in particular.

These concerns have led to proposals by the World Health Organization (WHO) such as the development of global strategies to contain the development of antibiotic-resistant bacteria that may adversely impact human health, and the banning of all uses of antibiotics from the same classes considered important in human medicine for purposes other than disease treatment (2). Although the antibiotic feed additives classed by the Food and Drug Administration Center for Veterinary Medicine (FDA-CVM) as "growth promoters" have been specifically targeted, other classes of antibiotics only used for treatment but belonging to classes considered important in human medicine have also been impacted. Sadly, scarcely documented concerns on the development of cross-resistance to the fluoroquinolone class of antimicrobials led to the loss of the most effective antibiotics that we had to treat bacterial infections in poultry, two antibiotics belonging to this class: sarafloxacin (Saraflox®) and enrofloxacin (Baytril®) were voluntarily withdrawn or banned in a legal battle, respectively (3,4). According to the latest published data from the National Antimicrobial Resistance Monitoring System (NARMS, 2007), the final ban of enrofloxacin in 2005 has not resulted in a reversal in the trend of increased fluoroquinolone-resistance in isolates of *Campylobacter jejuni* of human origin (6). In the mean time, many poultry flocks have suffered unnecessarily due to the lack of effective antibiotics for the treatment of systemic *E. coli* and fowl cholera infections while the producers have sustained devastating mortality losses that have ultimately impacted the viability of their operations.

Having said that, it is becoming increasingly apparent with the most recent unveiling by FDA-CVM of its Guide for Industry #209 (GFI-209) entitled "The Judicious Use of Medically Important Antimicrobials in Food-Producing Animals" that the future is likely to bring more restrictions and demand more veterinary oversight in the way antibiotics are used in animal agriculture (6). To that effect is important that all licensed poultry veterinarians are fully aware of the importance of using antibiotics for the prevention, treatment and control of disease in the most judicious and effective way possible in order to minimize the development of antibiotic resistance. In future years, prescription by a licensed veterinarian for all uses of antibiotics which are now obtained over-the-counter is very likely to occur.

This paper will concentrate on the key factors that poultry veterinarians must consider before they make the decision to prescribe or use an antibiotic, likewise this paper will review the practical aspects related to the use of antibiotics in poultry in order to maximize the potential for a successful outcome. Although technically speaking, sulfonamides (sulfas) are not considered antibiotics, they are considered as viable treatment options for bacterial infections of poultry.

DIAGNOSIS, ANTIBIOTIC, AND DOSAGE (DAD)

A good acronym to remember the three most important factors to consider when medicating a poultry flock with antibiotics is DAD, which stands for Diagnosis, Antibiotic and Dosage, if these three factors are properly addressed the potential for a successful therapeutic outcome increases.

ACCURATE DIAGNOSIS

The first, and perhaps most important factor in deciding whether or not to medicate a poultry flock with an antibiotic is obtaining an accurate diagnosis. The poultry veterinarian must have a high degree of certainty that he/she is dealing with a bacterial infection before instituting an antibiotic treatment, it is highly recommended that he/she collects samples for bacterial isolation and drug sensitivity testing or submit sick birds to the diagnostic laboratory prior to the initiation of treatment.

For example, if a poultry flock with increased daily mortality shows during necropsy of sick and fresh dead birds, widespread presence of a fibrinous exudate in serous membranes then a presumptive diagnosis of systemic *E. coli* or colispeticemia would be reasonable. If in addition, a substantial number of birds (>5%) are showing clinical signs of disease such as depression or anorexia or decreased feed consumption, then aseptic collection of samples for bacterial isolation and antimicrobial sensitivity testing or a submission of birds to the diagnostic laboratory and initiation of antibiotic treatment would be warranted.

It is important that poultry veterinarians educate service technicians on the proper use of antibiotics and emphasize that they should not be used in birds sickened by uncomplicated viral infections or suffering from other diseases such as coccidiosis or mycotoxicosis. Likewise, they must stress that antibiotics will not treat management deficiencies such as suboptimal brooding temperatures or insufficient ventilation.

CORRECT SELECTION OF ANTIBIOTIC

Once a presumptive diagnosis has been obtained and samples for bacterial isolation have been collected or birds submitted to the diagnostic laboratory, perhaps the second most important decision to be made is what antibiotic should be used.

Important considerations for the poultry veterinarian to consider are the suspected type of infection and the history of the farm in regards to previous response to antibiotics. The location of the infection is an important consideration for antibiotic selection, for example, an outbreak of necrotic enteritis may be treated with a water soluble antibiotic that is not systemically absorbed because the infection is limited to the intestinal tract, while an outbreak of systemic *E. coli* infection or colisepticemia requires the use of an antibiotic that is absorbed systemically where it can control the infection in internal organs and tissues other than the intestinal tract.

Surprisingly, even though colisepticemia is the most common systemic bacterial infection affecting turkeys none of the antibiotics approved for use of bacterial disease in turkeys administered through the drinking water have indications for its treatment. So the use of an antibiotic to treat the most common bacterial disease in turkeys is extralabel and therefore legally reserved or restricted by the order of a licensed veterinarian, this is perhaps one reason FDA-CVM is going to require more veterinary oversight in all future uses of antibiotics in food-producing animals. The clinical experience of the veterinarian and the laboratory history of drug sensitivity for the farm will aid in selecting an antibiotic with the greatest potential for a successful outcome.

Judicious use principles for the use of antimicrobials developed by the American Veterinary Medical Association and the American Association of Avian Pathologists indicate that whenever possible, an antibiotic of narrow spectrum should be used since broad spectrum antibiotics are more likely to create antibiotic resistance in bacteria that are not causing the infection (7).

CORRECT SELECTION OF DOSAGE

Once the antibiotic most likely to produce a successful therapeutic outcome has been selected, the next important factor to consider is the therapeutic regimen. This paper is dealing only with water-administered antibiotics, however, it should be kept in mind that for some antibiotics, feed administration is an option and it is always cheaper than water. However, birds usually stop eating before they stop drinking so a treatment regimen may require to start treatment with for example oxytetracycline in the water followed by switching to oxytetracycline in the feed once the birds have started to consume feed normally. This type of regimen is facilitated in poultry houses that can deliver feed from two feed bins.

Recommended label dosages for water-administered antibiotics are given in milligrams, grams, or units per gallon of drinking water but other labels suggest a fixed percentage of drug in the drinking water while others indicate milligrams per pound of body weight.

The author of this paper prefers dosing antibiotics based on body weight rather than a fixed amount or percentage per gallon of water as water intake is known to fluctuate widely due to a variety of health, environmental and dietary factors. In addition, younger birds drink a disproportionally larger amount of water per unit of body weight than older birds resulting in extreme fluctuations in drug intake per unit of body weight. For example, the manufacturer of Gallimycin® PFC shows in its label that dosing at 1 pack/130 gallons or at 1 pack/gallon of stock solution (when medicator is set to dispense 1 fl. oz./gallon) will

provide intakes in turkeys that vary from 3.1 to 34.2 mg/lb of body weight per day of erythromycin activity "depending upon age, class of turkey, feed conversion, environmental temperature, and relative humidity during medication period". That means that the dose that the turkeys are getting in terms of mg/lb of body weight can fluctuate as much as ten times! In the opinion of the author this type of fluctuation in dosage is unacceptable and that is why dosing on a mg/lb of body weight basis is a superior method.

As far as the frequency of administration and duration of treatment it is best that service technicians go by the label directions unless a licensed poultry veterinarian suggests an alternative. For example, "pulse dosing" for oxytetracycline and fluoroquinolones has been demonstrated to be an effective method to achieve quicker therapeutic levels of antibiotic in serum. In most cases pulse dosing involves mixing the calculated amount of drug that the flock should consume over a 24 h period into a smaller amount of stock solution (perhaps a quarter or third of the normal amount) so that the birds initially drink quicker a higher concentration of antibiotic in the drinking water to bring the serum concentrations of the antibiotic to a therapeutic level faster. In most cases, "pulse dosing" is recommended for the first day of treatment so that the antibiotic reaches therapeutic levels in the blood serum faster, and the therapeutic concentrations are subsequently maintained by regular daily water dosing (8). As this is an extralabel use, it requires consultation with a licensed veterinarian.

Most labels also detail limitations that include statements such as "consult a veterinarian or poultry pathologist for diagnosis", "add to drinking water", "prepare fresh solutions daily", "administer for seven days", "if symptoms persist after using this preparation for two or three days, consult a veterinarian", "do not use in laying hens producing eggs for human consumption", "solutions older than three days should not be used", "withdraw one day before slaughter", etc. All of these limitations printed on the product label should be followed in order to ensure the best potential therapeutic outcome possible.

OTHER IMPORTANT CONSIDERATIONS

In recognition of the veterinarian's knowledge of pharmacology and expertise in regards to drugs and antibiotics, the Animal Medicinal Drug Use Clarification Act, better known as AMDUCA, was approved in 1994 by the United States Congress (9). Essentially what AMDUCA does is allow licensed veterinarians to prescribe extralabel uses of certain approved animal drugs and approved human drugs for animals under certain conditions. The key constraints of AMDUCA require the prescribing veterinarian for any extralabel uses to have an established veterinarian-client-patient relationship and assurance that the extralabel use will not result in violations of tissue residues in food-producing animals, in addition, the prescribing veterinarian must comply with all regulations pertaining to AMDUCA published in the Code of Federal Regulations in which certain drugs are specifically prohibited for extralabel use, AMDUCA also specifically states that extralabel use does not apply to any drugs administered in feed.

There are other important considerations that cannot be covered during this presentation but that poultry veterinarians should always keep in mind such as differences in pharmacological effects and pharmacokinetics among antibiotics, cost of treatment and return on investment, solubility of the product, water quality and pH, undesirable chemical reactions due to residues of other drugs or incompatibilities between two compounds, improperly cleaned buckets for stock solution or mixing tanks. When considering cost of treatment, in the author's opinion is always better to spend more on a treatment if the desired clinical response or therapeutic outcome is achieved.

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AN ALTERNATIVE TO ANTIBIOTICS TO TREAT CELLULITIS AND SEPTICEMIA IN BROILERS

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SUMMARY

Colisepticemia is a common infectious disease found in poultry and it represents a substantial economic burden to Canadian poultry industry. Septicemia is defined as the invasion of the blood stream with the probability of infection of all tissues. Septicemia caused by *E. coli* has a significant economic impact due to the mortality that occurs and the fact the infected birds that survive often have significantly lower body weights compared with uninfected birds.

Avian cellulitis is a leading cause of meat condemnation and is a substantial economic burden to the Canadian poultry industry. It is easily contracted through scratches and skin punctures in the field making it very difficult to prevent. Despite the importance of combating this infection, no vaccines are currently available.

For decades, antibiotics have been used to treat bacterial infections in animals and humans, and are added to animal feed as growth promoters. The result has been the emergence of bacteria that are resistant to one or more antibiotics and this reality has serious implications for our ability to control illnesses in that occur during poultry protection. In addition severe limitations have been placed on the ability to use antibiotics in a prophylactic manner. As a consequence, livestock and poultry industries are searching for alternate methods of defence against illnesses and a replacement for prophylactic use of antibiotics in animal feed. We have sought to establish alternatives to antibiotics to treat *E.coli* induced septicemia and cellulitis in broiler chickens.

We have preliminary evidence suggesting that Host Defense Peptides (HDPs) together with an amino acid derivative called GB (known to promote wound healing) ameliorates clinical signs of avian septicemia in broiler birds. HDPs are naturally produced peptides many of which have inherent anti-bacterial, anti-viral and anti-parasitic activities (1-3). Further, they are expressed in epithelial cells and phagocytes at sites of infection wherein they can promote cellular recruitment of neutrophils, monocytes, mast cells, and T-helper (T_H) cells (1,4,5). They can also stimulate degranulation of mast cells, promote phagocytosis, induce the release of inducible nitrous oxide synthase to increase the rates of intracellular bacterial killing, stimulate tissue and wound repair, promote vascular repair, and suppress the stimulated release of pro-inflammatory cytokines such as TNF-α and IL-6 ((6-9), reviewed in (10)). Hence, HDPs provide a functional linkage between the innate and adaptive systems of immunity.

We have also tracked where HDPs migrate when injected into the birds (with or without infection) using unique dyes that fluoresce in the near-infrared region of light. Because animal tissue has extremely limited endogenous fluorescence within this region of light, these dyes allow unprecedented sensitivity when scanning tissues. Preliminary experiments have shown that these labels do not negatively influence peptide function, and scanning of birds renders useful information on peptide trafficking.

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(This research will be as submitted a full-length article to *Avian Disease* for publication at a later date.)

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BACILLUS LICHENIFORMIS PREVENTS NECROTIC ENTERITIS IN BROILERS AND IMPROVES PRODUCTION PERFORMANCE

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ABSTRCT

Three trials were conducted to evaluate the effect of *B. lichenformis* spores alone or in combination with an antibiotic growth promoter on broiler performance under commercial like condition. In all trials *C. perfringens* was administered as an intestinal challenge to produce necrotic enteritis (NE). The study consisted of three production trials in floor pens. The aim of the study was to test the effect of addition of *B. lichenformis* spores ($1{,}6x10^6$ CFU/g) alone or in combination with virginiamycin (15 g/t) or bacitracin methylene disalicylate (BMD) (55 ppm). In the first trial the birds were spray vaccinated for coccidiosis, and in the second and third trial salinomycin 60 g/t were added to the feed. In the first trial, using coccidiosis vaccine, the mortality of the challenge control group was 8% while in the second and third trial, using salinomycin the challenge control groups had a mortality of only 0.3% and 0.6% respectively. This indicates that the NE challenge model was not as effective using salinomycin compared to coccidiosis vaccine. In the first trial both the *B. licheniformis* group and virginiamycin group showed a significant ($P<0.05$) decrease in feed conversion ratio, increased weight gain, reduced NE lesion score and reduced NE mortality compared to the non medicated *C. perfringens* challenged group. In the second and third trial a significant ($P<0.05$) lower FCR and higher weight gain were seen in the group fed the *B. licheniformis* combined with the antibiotic growth promoter compared to the control and the antibiotic growth promoter group alone.

These studies demonstrate that the addition of *B. lichenformis* spores under commercial like condition with a mild NE outbreak can alleviate the negative effect of NE. Furthermore when *B. licheniformis* is administered in combination with antibiotic growth promoters it improves the broiler performance with regards to FCR and weight gain compared to the antibiotic growth promoter alone.

INTRODUCTION

Necrotic enteritis (NE) is the most common and financially devastating bacterial disease in modern broiler flocks (6). In 1977 Al-Sheikhly and Truscott (1) found that the intestinal necrosis characteristic of NE

was caused by the potent alpha-toxin produced by *C. perfringens.* Recently it has been found that theta toxin and other *C. perfringens* toxins may play a role in development of NE (2,4). The acute form of the disease leads to increased mortality in the broiler flocks (8). In the subclinical form of NE, damage to the intestinal mucosa caused by *C. perfringens* leads to decreased digestion and nutrient absorption resulting in reduced weight gain and increased feed conversion ratio (8).

Certain antibiotics such as BMD, tylosin phosphate, and virginiamycin are fed at sub therapeutic doses in the poultry industry as growth promoter. Part of the growth promotion properties results from the suppression of *C. perfringens* (8). *B. lichenformis* spores as probiotic or DFM has been reported to have the ability to prevent NE and could be an alternative to prophylactic use of antibiotics to overcome NE under commercial like condition (6). The aim of this study was therefore to evaluate the effect of *B. lichenformis* alone or in combination with an antibiotic growth promoter on broiler performance under commercial like condition with a mild NE outbreak.

MATERIALS AND METHODS

In this study a total of three *C. perfringens* challenge experiments were conducted.

Trial 1: Floor pen challenge production study with coccidiosis vaccine.

One thousand four hundred male Cobb X Cobb chicks were allocated to the study. All birds were spray vaccinated. At study initiation 50 males were randomly selected and allocated to one of four treatments: 1) Nonmedicated, noninfected 2) Nonmedicated, CP challenged 3) *B. licheniformis* spores (DSM 17236) at a dose of $1.6x10^6$ CFU/g of feed from d 0 to d 42, CP challenged, 4) Virginiamycin 15 g/t from d 0 to d 42, CP challenged.

Bird weights (kg) by pen were recorded at 0, 21, 35, and 42 d of age. An unmedicated corn-soybean meal based commercial broiler starter ration was formulated.

Necrotic enteritis was induced on 18, 19, and 20 d of age for all birds. Each bird was dosed via the feed with 1 mL (1.0×10^8 cfu/mL) of a fresh broth culture of *C. perfringens*, a field isolate of *C. perfringens* known to cause NE (3).

At 21 d of age, five birds from each pen were selected, sacrificed, group weighed, and examined for the degree of presence of NE lesions. The scoring was done blindly and based on a 0 to 3 score, with 0 being normal and 3 being the most severe (4). At the end of the trial (d 42) means for cage weight gain, feed consumption, feed conversion, NE lesion scores, and NE mortality were calculated (6)

Trial 2: Floor pen challenge production study with coccidiostat.

The second trial had the same setup as the first trial but with the use of 60g/t of salinomycin added to the diets instead of coccidiosis vaccine. The study had the following treatments: 1) Control, CP challenged 2) *B. licheniformis* spores (DSM 17236) at a dose of $8x10^5$ CFU/g and virginiamycin 15 g/t of feed from d 0 to d 42, CP challenged, 3) *B. licheniformis* spores (DSM 17236) at a dose of $8x10^6$ CFU/g and virginiamycin 15 g/t of feed from d 0 to d 42, CP challenged, 4) Virginiamycin 15 g/t from d 0 to d 42, CP challenged.

Trial 3: Floor pen challenge production study with coccidiostat.

The third trial had the same setup as the second trial. The study had the following treatments: 1) Control, CP challenged 2) *B. licheniformis* spores (DSM 17236) at a dose of $8x10^5$ CFU/g of feed and bacitracin methylene disalicylate, (BMD) 55 ppm from d 0 to d 42, CP challenged, 3) *B. licheniformis* spores (DSM 17236) at a dose of $8x10^6$ CFU/g of feed and BMD 55 ppm from d 0 to d 42, CP challenged, 4) BMD 55 ppm from d 0 to d 42, CP challenged.

In all three trials statistical evaluation of the data was performed using a STATISTIX for Windows program. The procedures used were General Linear procedures using ANOVA with a comparison of means using LSD (T) at 0.05 level of significance.

RESULTS

The results of the three trials are presented in Table 1. In the first trial, using coccidiosis vaccine, the mortality of the challenge control group was 8% while in the second and third trial, using salinomycin (60g/t), the challenge control groups had a mortality of only 0.3% and 0.6% respectively. This indicates that the NE challenge model was not as effective using salinomycin compared to coccidiosis vaccine.

In the first trial both the *B. licheniformis* group and virginiamycin group showed a significant decrease in feed conversion ratio, increased weight gain, reduced NE lesion score and reduced NE mortality compared to the non medicated *C. perfringens* challenged group. However there were no statistically difference ($P>0.05$) between the *B. licheniformis*, the virginiamycin or the non-challenge control group.

In the second trial a significant ($P<0.05$) lower FCR and higher weight gain were seen in the group fed the high dose *B. licheniformis* combined with virginiamycin compared to the control and the virginiamycin group.

There was no significant difference on mortality or NE lesion between any of the groups.

The third trial showed a statistically significant difference ($P<0.05$) with lower FCR and higher weight gain of the groups fed the *B. licheniformis* combined with BMD compared to the control and the BMD group. There was no significant difference on mortality between any of the groups. NE lesion was significantly ($P<0.05$) higher for the challenge groups compared to the non-challenge control group.

In conclusion, these studies show that the addition of *B. lichenformis* spores under commercial like condition with a mild NE outbreak can prevent NE. Furthermore when *B. licheniformis* is administered in combination with antibiotic growth promoters and it improves the broiler performance with regards to FCR and weight gain compared to the antibiotic growth promoter alone.

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Table 1. Result from three pen floor studies on feed conversion, weight gain, lesion score, and mortality.

Treatment	CP[1]	FCR (g/g)	WG (kg)	NE-lesion[2]	Mortality (%)
First trial (Coccidiosis vaccination)					
Control	-	1.862 c	2.444 a	0.0 c	0.0 b
Control	+	2.096 a	2.300 b	0.31 a	8.0 a
GPT 1.6E6	+	1.958 bc	2.386 ab	0.03 bc	3.4 b
Virginiamycin	+	1.911 bc	2.364 ab	0.17abc	1.7 b
Second trial (60 g salinomycin)					
Control	+	1.837 a	1.860 c	0.2 a	0.3 a
GPT 8E5+ Virg.	+	1.760 bc	1.946 ab	0.1 a	0.3 a
GPT 8E6+ Virg.	+	1.747 c	1.976 a	0.1 a	0.3 a
Virginiamycin	+	1.786 b	1.915 b	0.2 a	0.3 a
Third trial (60 g salinomycin)					
Control	+	2.055 a	1.925 c	0.28 a	0.6 a
GPT 8E5+ BMD	+	1.837 c	2.170 a	0.03 b	0.6 a
GPT 8E6+ BMD	+	1.824 c	2.210 a	0.05 b	0.0 a
BMD	+	1.915 b	2.092 b	0.13 b	0.0 a

Means in a column with no common letters are significantly different ($P<0.05$)

[1] *Clostridium perfringens* challenge (1.0 x 10^8cfu/mL)

[2] 0 = normal and 3 = most severe

USE OF ARTIFICIAL INTELLIGENCE (ARTIFICIAL NEURAL NETWORKS) TO CLASSIFY ANTIMICROBIAL RESISTANCE FROM *ESCHERICHIA COLI* SAMPLES ISOLATED FROM BROILERS

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Escherichia coli is common in poultry production and, is often found in the organism of animals and /or contaminating the litter on poultry farms. On the other hand, artificial intelligence, specifically artificial neural networks, is increasingly used as a tool to measure non-linear relations among variables. Studies on *E. coli* using artificial neural networks deal essentially with the genetic identification bases of DNA promoters (7), bacterial growth (3), predictions of mutagenicity, hepatotoxicity and teratogenicity (6), and others.

Escherichia coli is a microorganism known to cause avian diseases, although it is not clear whether it is a primary or secondary cause of the diseases attributed to it (5). Usually, *E. coli* is considered secondary to other agents, and it is the cause of avian extra-intestinal diseases. Many lesions are presented by poultry contaminated by this bacterium, such as colisepticemia, airsacculitis, peritonitis, pleura-pneumonia, pneumonia, swollen head syndrome, osteomyelitis, and others (5). According to Rocha (1) the virulence factors most frequently detected in respiratory isolates of E. *coli*, isolated in Rio Grande do Sul were serum resistance and colicins production.

Virulence mechanisms of *E. coli* isolates potentially pathogenic for broilers (APEC) have been continuously studied and it is supposed to be of multifactorial cause. Certain properties are primarily associated with these strains, and the most frequently mentioned include adhesion ability (*pap and fel*) production of colicins (*cva*), presence of aerobactin (*iut*), serum resistance (*iss*), temperature-sensitive hemagglutinin (*tsh*), and the presence of certain capsular antigens (*kps*) (4). The classification of genes that are pathogenic and/or associated with pathogenicity is very complex and a Pandora's Box. Unfortunately the process is not well characterized, i.e., it is not binary like black or white, but rather, it is constituted by shades of gray. This is applied to microorganism "pathogenicity" as well as to the "virulent" property of its genes. Thus, it is impossible to trace a precise line between virulent genes and other genes (11).

Souza, in 2006 (10), proposed an objective classification based on numerical results, which would make it feasible to analyze the data through statistical processing and enable comparison of results. In this classification the pathogenicity index has a range from 0 to 10.

Isolated PCR tests (uniplex) were used to identify seven genes associated with *E. coli* virulence (8,9). Recently, Fortes (2) studied the biochemical behavior of 261 samples of avian isolated *E. coli*. The results obtained showed that the pathogenicity indices (PI) in the samples positive for arginine, dulcitol, raffinose, and sucrose are significantly higher than in those of the negative ones. On the other hand, negative isolates for salicin and for the indole test also have significantly higher PIs than the positive ones. He also concluded that salicin, sucrose, raffinose, adonitol, and dulcitol, as well as arginine and ornithine, presented variable results for *E. coli*.

The purpose of this study is to construct artificial neural networks to predict the results of the antimicrobial resistance of 246 isolates of *Escherichia coli* from poultry production. The inputs used are already existing information obtained from previous studies generated at CDPA-UFRGS, which now constitute a solid database with information on the samples isolated from poultry.

MATERIALS AND METHODS

The data used in this study are collected in a database generated at the CDPA (Center of Diagnosis and Research in Avian Pathology), Veterinary Faculty, Federal University of Rio Grande do Sul. Information on 246 isolates of *Escherichia coli* was used as follows:

- Biochemical characteristics (adonitol, arginine, dulcitol, ornithine, raffinose, sucrose,

salicin);

- Pathogenicity indices (0 to 10);
- Lesions caused by inoculation in one-day old chicks (cellulitis, peritonitis, perihepatitis, pericarditis, and airsacculitis);
- Characterization of genes associated with pathogenicity (*papC, felA, cvaC, iutA, ISS, tsh* and *kpsII)*;
- Antimicrobial resistance to 14 antibiotics (amikacin, amoxicillin/clavulanic acid, ampicillin, cephalexin, ceftiofur, cefuroxime, ciprofloxacin, clindamycin, cotrimoxazole, enrofloxacin, gentamicin, norfloxacin, ofloxacin and tetracycline);
- Origin of the isolates (broiler litter, cellulitis and respiratory pictures); and
- Bacterial motility.

The artificial neural networks were constructed using the Neuroshell Classifier 2.1 software (Ward Systems Group, Inc., Frederick, MD, USA, 1997-2000).

The results available were transformed into a binary form. Thus, the negative results are represented by number 0 and the positive ones by 1. This criterion was used for sensitivity (0) or resistance (1) to the 14 antibiotics studied, to the absence (0) or presence (1) of genes associated with pathogenicity, to the absence (0) or presence(1) of the different lesions observed in the chicks. Non-motile isolates were represented by 0 and motile isolates by 1. As to the origin of isolates, it was necessary to add one more value and the data was noted as follows: litter (0), cellulitis (1) and respiratory pictures (2).

The inputs chosen to construct the artificial neural networks were pathogenicity indices, lesions induced in one-day old chicks, characterization of the genes associated with pathogenicity, biochemical behavior (only biochemicals that presented behavioral variation in the isolates were utilized. They were adonitol, arginine, dulcitol, ornithine, raffinose, salicin, and sucrose), and the origin and motility of the isolates. The outputs were resistance or sensitivity to l4 antibiotics obtained by Salle *et al.*, (2009).

RESULTS AND DISCUSSION

Table 1 shows the characteristics of the artificial neural networks constructed with the inputs Biochemical Behavior (B), Genes associated with pathogenicity (G), Pathogenicity Index (PI), *E. coli* motility (M), Lesions produced in one-day old chicks (L) and Origin of Isolate (O). The outputs are the antibiotics studied. It is also clearly seen that the artificial neural networks were capable of classifying bacterial resistance to the antibiotics studied. Amoxicillin had 100% correct classifications and used only four data inputs: Genes associated with pathogenicity, biochemical behavior, pathogenicity index, and isolates motility. The other isolates required further information, such as origin of the isolates and the lesions that they induced when inoculated in one-day old chicks.

The same table shows that the correct classifications had a range of 90.4% to 100%, which configures it in an excellent tool to infer the behavior of a bacterial isolate to a given antibiotic. When the sensitivities of the classifications were analyzed, the range was 84% to 100%, for the intermediate isolates, 81% to 100% for the resistant ones, and 89% to 100% for the sensitive ones. As to the specificities, the results were 100% for the intermediate ones, 90% to 100% for the resistant ones, and 81% to 100% for the sensitive ones.

It should be emphasized that the information on the genes used in this experiment refers to those reported in the literature as associated with isolate pathogenicity and not with antimicrobial resistance. Even so, knowledge about their presence or not, associated with other information used as inputs to construct the artificial neural network, was able to predict what the antimicrobial resistance of the isolates would be. This observation recalls the references of Wassenaar and Gaastra, (11), in which attention was called to the complexity of the definition of pathogenic genes and those associated with pathogenicity, leading to think that the combinations among the different genes present in the bacteria are probably not linear.

This methodology allows simulation by modifying the input data to obtain the desired output. Finally, it was demonstrated that the artificial neural networks can learn to classify the bacterial resistance of the isolates used in the experiment, based on the inputs that were chosen. In the references consulted, no paper was found discussing the topic from the perspective presented here.

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Table 1. Classification of bacterial resistance (intermediate, resistant or sensitive) of 246 *Escherichia coli* samples isolated from broilers to 14 antibiotics using artificial neural networks.

OUTPUTS	INTPUTS	%COR*	(i) S**	(r) S	(s) S	(i) E	(r) E	(s) E
Amikacin	G+B+IP+M+L	100.0	1.0	1.0	1.0	1.0	1.0	1.0
Amoxicillin	G+B+IP+M	100.0	1.0	1.0	-	1.0	1.0	-
Ampicillin	G+B+IP+M+L+O	96.34	1.0	0.9	0.98	1.0	0.989	0.91
Cephalexin	G+B+IP+M+L+O	100.0	1.0	1.0	1.0	1.0	1.0	1.0
Ceftiofur	G+B+IP+M+L	99.59	0.91	1.0	1.0	1.0	1.0	0.94
Cefuroxime	G+B+IP+M+L	100.0	1.0	1.0	1.0	1.0	1.0	1.0
Ciprofloxacin	G+B+IP+M+L	96.34	0.84	0.92	0.99	1.0	0.98	0.92
Clindamycin	G+B+IP+M+L+O	100.0	1.0	1.0	1.0	1.0	1.0	1.0
Cotrimoxazole	G+B+IP+M+L	90.24	1.0	0.91	0.89	1.0	0.90	0.91
Enrofloxacin	G+B+IP+M+L+O	98.78	0.97	0.97	1.0	1.0	1.0	0.97
Gentamicin	G+B+IP+M+L+O	95.93	-	0.81	0.99	-	0.99	0.81
Norfloxacin	G+B+IP+M+L	98.37	0.90	0.94	1.0	1.0	1.0	0.92
Ofloxacin	G+B+IP+M+L	99.19	0.89	1.0	1.0	1.0	1.0	0.96
Tetracycline	G+B+IP+M+L	94.72	1.0	0.94	0.96	1.0	0.96	0.94

* Percentage of correct classifications;

** (i) S= sensitivity for isolates with intermediate resistance; (r) S= sensitivity to resistant isolates; (s) S= sensitivity for sensitive isolates; (i) E= specificity for isolates with intermediate resistance; (r) E= specificity for resistant isolates; (s) E= specificity for sensitive isolates.

USE OF ARTIFICIAL INTELLIGENCE (ARTIFICIAL NEURAL NETWORKS) TO CLASSIFY THE PATHOGENICITY OF *ESCHERICHIA COLI* ISOLATES FROM BROILERS

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INTRODUCTION

The virulence mechanisms of *E. coli* isolates potentially pathogenic for broilers (APEC) have been continuously studied and it is supposed to be of multifactorial cause.

Certain properties are primarily associated with these strains and the most frequently mentioned include adhesion ability (*pap* and *fel*), production of colicins (*cva*), presence of aerobactin (*iut*), serum resistance (*iss*), temperature sensitive hemagglutinin (*tsh*), and presence of some capsular antigens (*kps*) (5).

However, what sets apart virulent and avirulent strains remains a problem in diagnosis and consequently, in the decision-making by the field veterinarian. Probably, this still happens because of the complex interactions that exist between the virulence factors of *E. coli* and the fact that conventional procedures for determining the pathogenicity of *E. coli* in which animals are inoculated are time consuming, expensive, and ethically challenged by the international community. Moreover, in determining the *in vivo* pathogenicity index, there is no standardization of procedures and classifications, which prevents the comparison of results. In an attempt to solve these problems, an objective classification was proposed based on numerical results, which make possible the analysis of data using statistical analysis and allows the comparison of results. In this classification, the pathogenicity index ranges from 0 to 10 (10).

Molecular diagnostic methods have evolved significantly, and the diversity of genetic profiles found in works available in the literature suggests that there is real interaction between the virulence factors of *E. coli*. However, attempts to establish virulence markers have not yet resulted in significant progress. In this study, the option for checking the presence of *papC*, *felA*, *cvaC*, *iutA*, *iss*, *tsh*, and *kpsII* genes is due to the difference in the frequency of these genes among strains isolated from diseased broilers in comparison with those from clinically healthy broilers or feces (1,4,8)(2)(3)(6).

We believe that one of the tools that has the potential to aid in the resolution of ethical problems involving the use of animals and to establish a diagnosis that includes the pathogenicity index of *E. coli* is the use of mathematical modeling, particularly artificial neural networks. Works that use neural networks to predict the pathogenicity of bacteria were not found in the literature; however, several authors have attempted to establish markers of virulence and trace models that include the interaction of the main factors.

The objective of this paper is to show that it is possible to perform pathogenicity prediction through the use of artificial neural networks, without using animals and, thus, provide field veterinarians with reports including the degree of pathogenicity of isolates so that their decisions can be more objective and accurate.

MATERIAL AND METHODS

Data base. The data analyzed refers to the presence of genes responsible for adhesion ability, fimbria P (*papC*) and fimbria F11 (*felA*), production of colicins (*cvaC*), presence of aerobactin (*iutA*), serum resistance (*iss*), temperature sensitive hemagglutinin (*tsh*) and presence of capsular antigens K1 and K5 (*kpsII*), pathogenicity index or classification (10), isolates motility, and e origin.

A total of 293 *E. coli* isolates were analyzed. Seventy-six litter isolates, 159 cellulitis lesions and 58 colisepticemic broiler organs. The isolated strains of litter and cellulitis lesions were isolated from the same batches coming from 73 different farms. Pictures of colisepticemia came from 55 different batches of broilers in which breathing symptoms were observed and the necropsy revealed lesions compatible with colibacillosis.

Networks. The artificial neural networks were constructed through the use of Neuroshell Classifier 2.1 software (Ward Systems Group, Inc., Frederick, MD, USA).

The networks construction was performed in two separate phases: training and validation.

Three networks will be presented, in which the input layer includes information on the presence of *papC, felA, cvaC, iutA, iss, tsh* and *kpsII* genes, isolates motility and origin (colisepticemia, cellulitis and litter). The output layer was formed by the "Pathogenicity Index (PI)" (Souza, 2006). In the first network, PI varies from 0 to 10. In the second one, PIs were grouped in three classes or categories: apathogenic/low pathogenicity (A/LP) with PI between 0 and 3.99; intermediate pathogenicity (IP) refers to PIs between four and 6.99; and high pathogenicity (HP) with PI ranging from seven to 10. Finally, a third network was constructed, in which daily situations experienced by field veterinarians were represented and bacteria were considered as of low pathogenicity (apathogenic/low pathogenicity (A/LP) - PI between 0 and 3.99) or pathogenic (intermediate/high (IP/HP)-PI between four and 10). Table 1 summarizes these three networks.

We have called “input layer” the set of variables presented for model calculation and “output layer” the variable to be predicted.

RESULTS AND DISCUSSION

According to the results of Network 1 in which the PI range from 0 to 10 was fully used and generated 11 data categories, the correct PI classification at training phase was 54.27% (160/293), in validation was 49.83% (146/293). However, the specificity of nine out of 11 data categories was higher than 95%. Sensitivity had a wide variation range, from 16 to 86% and the prediction success rate, per category, was between 16 and 80%. We believe that this data reflects four factors: low amount of isolates per category, high diversity of genetic profiles (73), the fact that isolates with same profile presented different PIs and isolates classification in adjacent or close categories. In category "0," 73% of errors classified the isolates as belonging to category "1." In "1," 89% of isolates were classified as "0." In "2," 86% were classified between categories "0" and "1." In category "3," 74% were classified as "0" or "1." In "8," 14% were classified as "7" and 57% as "9" or "10." In "9," 55% were classified as belonging to categories "7" and "10." In "10," 75% were concentrated in category "9." In categories "4," "5," "6," and "7," errors were concentrated in categories "0" and "1." In "4," 53%; in "5," 70%; in "6," 50%; and in “7,” 74%.

Since this paper always pursued the highest accuracy of the models generated, we tried to solve the problems concerning low amount of isolates per category and isolates classification in adjacent or close categories, reducing to three the number of categories and creating Network 2: Apathogenic/low pathogenicity (A/LP) with PI between 0 and 3.99; intermediate pathogenicity (IP) with PI ranging from four to 6.99; and high pathogenicity (HP) with PI between seven and 10. In this case, the correct classification of isolates for the training phase was 80.55% (236/293) and for validation was 76.45% (224/293). The lower success rate (41%) occurred in the intermediate category. An analysis of the distribution of errors showed that 40% of isolates were classified as of high pathogenicity and 60% as of low pathogenicity, thus confirming the classification of this category as intermediate.

As expected, the reduced amount of categories improved Network performance. In an effort to further improve its performance, we started to work with only two categories. Thus, Network 3 was configured as follows: apathogenic/low pathogenicity (A/LP) with PI between 0 and 3.99 and intermediate pathogenicity/ high pathogenicity (IP/HP) with PI ranging from four and 10. This reduction in the amount of categories also meets the needs of field veterinarians and makes their decisions easier because the measures to be adopted in relation to *E. coli* isolates of intermediate or high pathogenicity are the same. The same occurs with apathogenic or low pathogenicity strains. With this configuration, the correct classification of isolates for training phase was 83.96% (246/293) and for validation was 81.2% (237/293). Predictions with similar percentage were made for mutagenicity, hepatotoxicity, and teratogenicity of thiophene derivatives, with predictions between 80 and 85% (7); and in studies of different class promoters (DNA sequence), with many functions in *E. coli*, it is reported a success level between 63 and 82% (9). It confirms that the models generated are of good quality, both for the level of discovery and for the improvement in relation to the previous situation.

CONCLUSION

By reaching correct classification percentages higher than 80%, the method proposed here has proved to be a powerful tool to support the veterinarians’ decisions in the field. The characteristics of this model allow the classification of isolates pathogenicity in broiler houses with a good degree of reliability, considering the sensitivity and the specificity.

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Table 1. Inputs and output of artificial neural networks for classification of the pathogenicity index of *Escherichia coli* isolates.

Network Identification	Input	Output
1	Genes*, motility, origin of strains	PI (0-10)
2		A/LP(0-3.99), IP(4-6.99), HP(7-10)
3		A/LP(0-3.99), IP/HP(4-10)

**papC*, *felA*, *cvaC*, *iutA*, *iss*, *tsh* and *kpsII*

OUTBREAKS OF AVIAN ENCEPHALOMYELITIS WITH UNUSUAL LESIONS IN YOUNG BROWN CHICKENS

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Avian encephalomyelitis (AE), also called "epidemic tremor," is an infectious viral disease of chickens, turkeys, quail, and pheasants. AE is caused by Picornavirus and recently it has been placed in a new genus, *Tremovirus.* AE is characterized by neurological signs such as ataxia and paralysis in young chickens and transient drop in egg production in layers. Survivors often develop cataracts later in life. Morbidity can range from 40 to 60% and mortality from 25 to 50% depending on whether the chicks came from immune birds or not. There are no significant gross lesions due to AE except for pale areas in the muscular layer of the gizzard occasionally. Microscopically there is often disseminated non-suppurative encephalomyelitis and mild to severe infiltration and aggregation of lymphocytes in the

muscular layers of the proventriculus and gizzard. Other lesions such as pancreatitis, myocarditis, and myositis have also been described occasionally. AE has been well controlled by proper vaccination of the breeder chickens.

Two outbreaks of AE occurred in brown chickens around 13 d of age on the same ranch about one year apart. History of the chicks included neurological signs and increased mortality and morbidity in the flock. Eighty live chicks from flocks of 10,000 and 15,000, respectively, were examined in the laboratory. Clinical and laboratory findings including serology and pathology were followed in the chicks at different ages, 13 to 56 d. Clinically most of the chicks exhibited ataxia and down on legs in all age groups, but head tremors were most prominent in chicks between 13 and 28 d of age. There were no significant gross lesions. Microscopically, lesions of AE characterized by encephalomyelitis, proventriculitis, and ventriculitis (gizzard) were observed in most of the chicks. Other lesions such as mild to severe primarily lymphocytic pancreatitis, myocarditis, and myositis were also observed. Unusual lesions comprised of neuritis of the peripheral nerves; ganglioneuritis of the myenteric plexus in proventriculus, gizzard, and esophagus and spinal ganglia; eye lesions (iridocyclitis, choroiditis, and pectenitis); ear lesions (otitis interna and externa); adrenalitis; splenitis; nephritis; leiomyositis of the intestine; and osteomyelitis. In addition mild to severe cataracts were observed in a few chicks as young as 13 d of age. A few chicks also had vasculitis of the arteries in the muscular layer of the gizzard. Serologically titers for AE were low positive in younger birds but increased in older birds. The cause of AE outbreak in the flocks was determined to be due to improper vaccination of some of the breeder chickens.

ENVIRONMENTAL STRESS AND FEED ADDITIVES REGULATES *SALMONELLA* SURVIVAL AND HOST ASSOCIATION

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SUMMARY

Salmonella is an important food borne pathogen throughout the world that leads to gastroenteritis through out life in humans. It is often associated with animals destined for the human food supply as a common commensal microbe. However, it is increasingly common to find *Salmonella* in leafy greens and processed ready-to-eat products. The mechanisms used by *Salmonella* to persist throughout the food chain are not clearly established. This study examined environmental stress, prebiotic and probiotic supplementation to reduce *Salmonella* survival and host association, respectively. Only osmotic stress significantly (P<0.05) decreased survival. Pre-treatment with peroxide shock, osmotic shock and osmotic stress significantly (P<0.05) reduced survival during subsequent acid stress. Exposure to only cold stress rescued the cell from subsequent acid stress and enabled *ST* (ST) to persist during cold stress for at least 336 h. Cold stress also increased adhesion of ST to colonic epithelial cells *in vitro*. Addition of prebiotic oligosaccharides changed the adhesion and invasion of ST and S. Enteritidis (SE) with varying success to block association. Use of probiotic lactic acid bacteria successfully reduced *Salmonella* adhesion, but the reduction was specific to the serovar and probiotic organism used. The complex interaction between stress, prebiotic addition, and probiotic interactions was specific to each serovar tested. This study implies that use of these treatments to reduce *Salmonella* is likely, but it needs to be tailored to the serovar of interest.

INTRODUCTION

Salmonella enterica commonly causes gastroenteritis in humans that arises primarily from the food supply. Population-based surveillance for bacterial food borne infections with laboratory confirmed cases in 2008 found that *Salmonella* accounted for ~140 cases/100,000 human infections (www.cdc.gov/foodnet), with ST accounting for ~21 cases/100,000 infections. Two nation-wide outbreaks caused by *ST* in 2006 and 2008 linked to contaminated tomatoes and peanut butter, respectively, led to

enormous economic loss (13) and 714 confirmed cases of salmonellosis as of April 2009 according to CDC. These outbreaks along with the outbreaks associated with eggs in 2010, and the long history of persistent problems with food borne *Salmonella* and *Campylobacter* prompted the US government to re-examine the entire food inspection service and protocols in the USA leading to new legislation in 2010 that calls for additional inspection and tighter controls on infectious agents during production and processing.

Use of traditional food processing conditions (osmotic, oxidative, acid, temperature) becomes even more important with the recommendation to reduce salt consumption and addition to food. While refrigeration (5 to 14°C) is the most commonly used method to increase the shelf life of the food, it often has little effect on the total bacterial load. *Salmonella* have efficient mechanisms to survive processing and persist in the environment to cause gastrointestinal disease in humans. *Salmonella* are well document to survive acid (1,11), peroxide (4,14), osmotic (2,5-7), and heat stress (12,15) with many of the genes responsible for the survival well defined. Exposure to slightly acidic conditions protects the organism against subsequent severe stressful conditions (8-9,10), leaving the food supply vulnerable to long-term persistence of *Salmonella* in the food chain. In addition to stressful conditions during its life cycle during transit between hosts (i.e. in the environment) *Salmonella* must overcome extreme stresses and defense mechanisms encountered during consumption and infection. Upon ingestion of the contaminated food, *Salmonella* encounters stomach acidity as host's first line of defense. There is initial lag period of 90 min (3) before gastric emptying starts, and pH of the stomach after consumption of food is between pH 3.0 – 5.0. Thus in typical conditions, *Salmonella* experiences stresses in environment or during food processing followed by stronger acid stress upon ingestion of contaminated food. Hence, we hypothesized that pre-adaptation to stresses encountered during journey from environment to food processing facility induces survival of *Salmonella* in the stronger acidic (pH 4.0) environment. This study examined cellular stress, inclusion of prebiotic formulation, and probiotic addition to modulate *Salmonella* survival and host association.

MATERIALS AND METHODS

Bacterial strains. *Salmonella enterica* serovar Typhimurium LT2 ATCC 700720 (ST) and *Salmonella enterica* serovar Enteritidis (SE) were used in this study.

Stress treatments. Stress treatments were divided into shock (30 min) and stress (5 h). In each treatment log phase cells were treated with cold (5°C), oxidative (1 mM H_20_2), acid (pH 5.3), and osmotic (5% NaCl). During the stress treatment each condition was sampled to determine the viable cell count with pour plate method using nutrient agar (Difco). Sequential double stress was done by pre-treating the bacteria with each stress followed by exposure to acid (pH 4.0) for 90 min. The viable cell count was determined using pour plates of nutrient agar (Difco).

Host association. ST was used to infect fully differentiated human colonic cells (caco2) with an MOI of 1000. The adhesion and invasion were determined using qPCR after the treatment of the cell line with prebiotic preparations and lactic acid bacteria used as probiotics in animals and humans.

RESULTS

Stress survival. To understand the survival of ST, we examined the effect of abiotic stresses encountered during in the environment as well as host association in humans and animals. The initial experiment examined the ability of ST to survive cold stress for at least 336 h to test the hypothesis that *Salmonella* can withstand long periods of exposure to low temperatures during the transition between animal hosts in the environment. ST survived cold stress for at least 336 h (Fig. 1A). During this time course, survival declined after 48 h, while at 5°C a significantly (P<0.05) high number of viable bacteria were observed after 240 h. This observation led us to suspect that ST may survive other abiotic stresses from the environment as well. Exposure to peroxide, osmotic, and acid for 0.5 h (shock) and 5 h (stress) was examined to find that only peroxide shock significantly reduced survival (P<0.05) (Fig. 1B).

Since cold and acid treatments did not inhibit survival, we investigated each treatment in combination with subsequent acid stress to mimic environmental transit followed by consumption of *Salmonella* during a meal where it would encounter a secondary acid stress in the stomach. Gastric transit of ~90 min (4,5) exposes organisms to a pH ~4.0 initially upon consumption of food. Consequently, this study investigated survival after pre-exposure to abiotic stress followed by exposure to secondary acid stress of pH 4.0 for 90 min. Pre-exposure to peroxide shock, osmotic shock and osmotic stress followed by exposure to acid stress (pH 4.0 for 90 min) significantly (P<0.05) inhibited survival, while pre-exposure to cold stress (5°C for 5 h) with subsequent acid stress (pH 4.0 for 90 min) significantly (P<0.05) induced survival as compared to survival upon exposure to acid stress (pH 4.0 for 90 min) alone (Fig 1C). Pre-exposure to cold stress rescued the cells from effects of acid stress (Fig 1D). These observations demonstrate the complex

network of gene regulation and metabolic proteins used for survival induced by cold and acid stress, while the combination of osmotic/acid successfully inhibited ST by ~2 decades with increasing exposure time.

Prebiotic and probiotic blocking. Surprisingly, cold stress increased adhesion of *Salmonella* to colonic epithelial cells using *in vitro* testing (Fig. 2). In and effort to reduce adhesion of stressed cells we examined six prebiotic and five probiotic treatments. Each treatment had a unique effect on ST and SE adhesion. A dose response was observed with each prebiotic, but in a few treatments addition of the same prebiotic had the opposite effect between ST and SE, e.g. MOS_1 and MOS_3. In fact, MOS_3 encouraged adhesion of SE beyond that of the control.

Addition of probiotic lactic acid bacteria was tested to reduce adhesion (Fig. 2). Again, probiotics reduced adhesion of ST and SE in different amounts between the treatments. For example, *Lactobacillus acidophilus* NCFM significantly reduced ST adhesion, but had no effect on SE adhesion, while *Lactobacillus casei* ATCC334 had a similar effect on both serovars.

In additional studies we determined that Bifidobacterium infantis (BI) had a beneficial effect on epithelial cells. To determine if this effect was possible in the presence of ST we examined co-infection with two different treatment types. Only a slight decrease was observed if ST was added 15 min. before addition of BI. If BI was added first or if BI and ST were added together a significant (P<0.5) was observed in adhesion and invasion.

CONCLUSION

Stress response is a critical parameter that enables *Salmonella* to survive food processing and environmental transit. Cold shock and stress enabled ST to resist acid stress and increase adhesion to colonic epithelial cells. Only osmotic stress consistently reduced the viability and did not protect the cell from subsequent acid stress. Use of pre- and probiotics to reduce adhesion of ST and SE was variable, and in some cases increased association *in vitro*. Understanding of the underlying mechanisms of stress resistance and host association will provide additional strategies to further reduce *Salmonella* viability to limit transit in the environment and limit host association.

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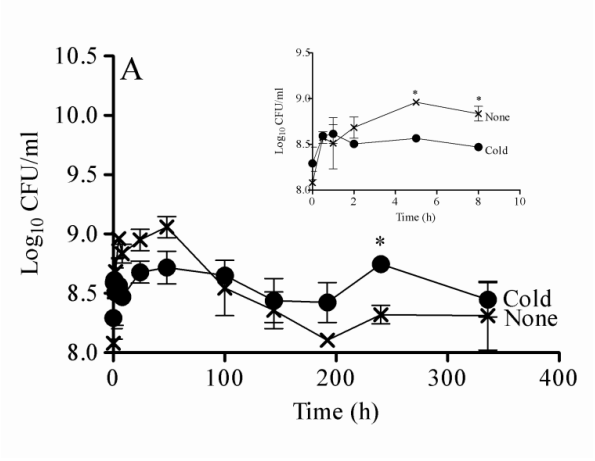
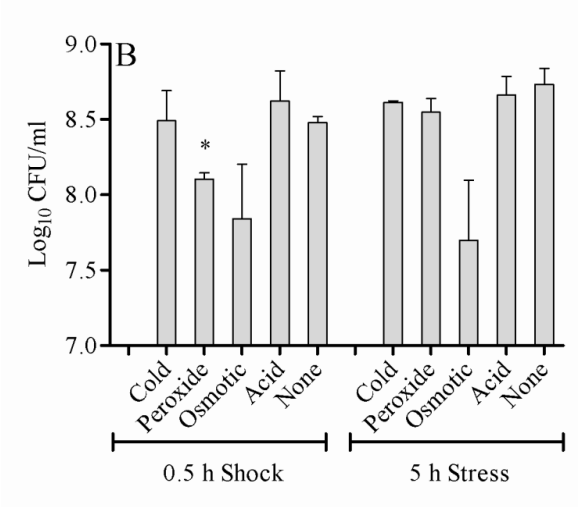
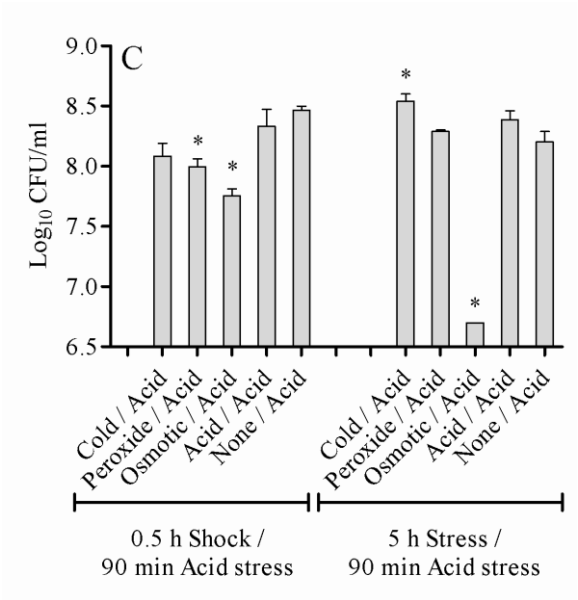
Figure 1. Survival of ST during stress treatments. Panel A depicts survival of ST during cold stress (5°C) for extended period of time. Inset: Enlarged for growth between 0 h – 8 h. Panel B depicts survival of ST upon stress treatments for 0.5 h and 5 h. Panel C depicts survival of *ST* upon stress treatments followed by acid stress (pH 4.0 for 90 min). Panel D depicts survival upon cold stress for 5 h and upon cold stress for 5 h followed by acid stress for 90 min. The asterisk (*) represents the significant difference (P<0.05) as compared to the control.







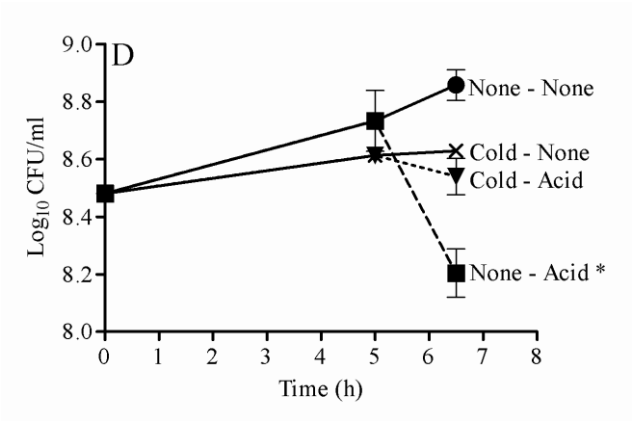
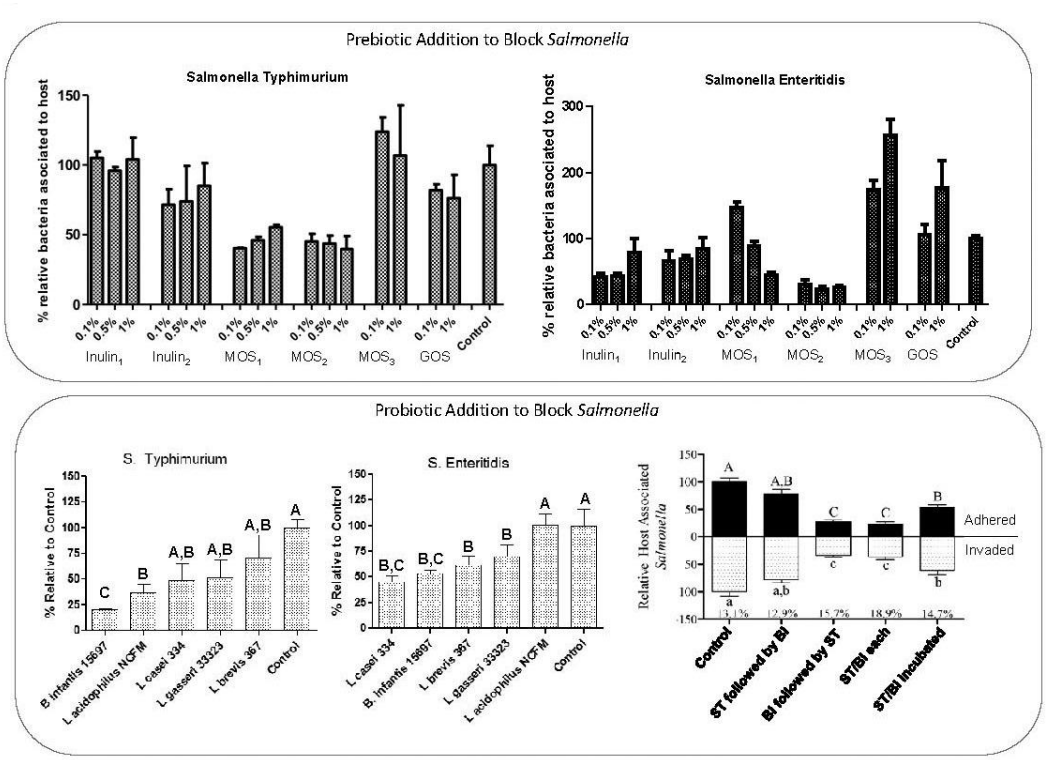


Figure 2. Adhesion and of ST and SE to differentiated colonic epithelial cells (caco2) during pre- and probiotic addition. Commercial sources of the prebiotic treatments were obtained and used at increasing concentrations prior to addition of ST or SE. Probiotic treatment was done using mid-log phase lactic acid bacteria with addition 15 min prior to adding ST or SE. Invasion assays were done 60 min after infection.



PERFORMANCE EVALUATION OF *SALMONELLA* PCR SCREENING ASSAYS IN POULTRY SAMPLES

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Increased regulations and consumer demand for enhanced *Salmonella* testing in commercial poultry has created a need for high-throughput, fast and accurate alternatives to conventional culture methods which can take up to two weeks for a negative test on a delayed secondary enrichment test (vs. 24 h in PCR). We evaluated real-time PCR tests that our lab has been using to screen for *Salmonella* spp. in various broiler live production environmental and processing plant samples (including carcass rinses and further processed parts). When testing broiler carcass rinse samples w/arguably low counts and possibly injured *Salmonella* cells, the sensitivity of the ADIAFOOD Real-Time PCR and DuPont Qualicon Bax PCR tests ranged from 88-97% while specificity varied from 98.9 to 100%. In different types of samples tested, there seems to be closer percent agreements (90-100%) between PCR and conventional culture in samples that are traditionally recognized to have a higher prevalence and amount of *Salmonella* (chick tray, cloacal swabs, certain batches of carcass rinses, comminuted meat). A pattern of lower agreement is seen in samples containing less viable *Salmonella*. This is probably due to prolonged holding times, hence, longer contact time to residual sanitizers in the carcass rinse (older carcass rinses: 70%) and low water activity (litter drag swabs: 22%).

In our recent *Salmonella* Enteritidis shedding and egg deposition study, DuPont Qualicon Bax PCR showed very high sensitivity/inclusivity and specificity/exclusivity (100%) when testing voided feces from experimentally infected (10^8 cfu/mL oral gavage) SPF-leghorns. Moreover, PCR specificity is 100% in internal egg contents. *Salmonella* was not detected by PCR and culture in all internal eggs contents tested; hence, sensitivity cannot be evaluated. Overall, the excellent test performance might be positively influenced with the arguably higher prevalence and amount of *Salmonella* in the voided feces, as well as the freshness/viability of the fecal samples of experimentally infected leghorns. The incidence of false-positives is remarkably low, as evidenced by the high specificity rates regardless of sample type. Both PCR tests have demonstrated their utility as screening tools for *Salmonella* broiler and layer samples to reduce workloads and shorten turn-around time for *Salmonella* testing.

DYNAMIC IMAGING TELEPATHOLOGY (INCLUDES LIVE DEMONSTRATION)

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Advances in computer technology have made real time sharing of histopathology over the internet, an affordable option. Telepathology has become an important aspect of telemedicine in the past few years and this trend will continue in the years to come. The first telepathological system was developed in the 1960's at the Massachusetts General Hospital. However, it was not explored and used until the late 1990's (2). Telepathology is the viewing of pathological specimens on monitor when away from the microscope. There are two main types of imaging telepathology: static and dynamic.

In the Static Imaging Telepathology (SIT), pathologists select images, store them on a computer, and upload the image to other pathologists. SIT is has the benefits of being the most reasonably priced and usable systems. The major downsides of this technology are that a small number of images can be transmitted and that the consulting telepathologist is unable to select the images he/she wants transmitted. Deficiencies in focus are also commonly identified.

An adaptation of SIT is virtual slide telepathology. Virtual slide systems utilize automated digital slide scanners that create a digital image file of an entire glass slide (whole slide image). This file is stored on a computer server and may be navigated at a distance, over the Internet, using a browser. However, high throughput virtual slide scanners (those producing one virtual slide or more per minute) are currently expensive. High quality, virtual slide digital files are relatively large, and requires the maintenance of large storage capacity. Storing and simultaneously retrieving large numbers of telepathology whole slide image files can be cumbersome, introducing their own workflow challenges in the pathology laboratory. Virtual slide systems require technical staff to establish and maintain the computer infrastructure and database software.

Dynamic Imaging Telepathology (DIT), also known as "Real Time Video Imaging," involves the use of a microscope along with a personal computer to send images over the internet. Web-based telepathology incorporating video streaming is now possible and allows collaboration between users. Major advantages of telepathology include providing immediate access to subspecialty pathology consultants, generating second opinions, and assisting pathologists in completing or refining a differential diagnosis. Multiple studies of the diagnostic accuracy of telepathology for evaluation of paraffin-embedded permanent sections have shown high agreement between conventional light microscopy and remote telepathology diagnoses (1,4) and higher than when using SIT (3). Real-time systems perform best on local area networks (LANs), but performance may suffer if employed during periods of high network traffic or using the Internet proper as a backbone.

The major biological and scientific microscope companies (Leica, Nikon, Olympus, and Zeiss) offer DIT systems. All camera systems are controlled by computer software, allows for several measures, and have a database functionality to organize photos. In most cases the camera needs to be connected to a Windows computer (not Apple or Unix); but Nikon and Zeiss offer standalone digital imaging systems that eliminate the need for a computer connection. This means that the system operates independently from an operational system, with no need for video cards

installation or special computer interface. While Nikon uses the digital camera-controller (DS-L2) mounted on a standard microscope, Leica (DMD108) has an integrated microscope-camera designed without eyepieces.

Carl Zeiss has a unique system (AxiVisio) that can use either a consumer camera or a microscope scientific camera with the microscope. The Olympus system (CellSens) allows for the up to 50 sites to be connected at one time to review images over the internet, while the other systems allow for smaller groups. DIT systems are currently relatively affordable, the price for the camera, adaptors and software are typically in the region of $10,000 (the microscope is additional).

The most significant advantage offered by DIT is the potential for immediate access to a pathologist at a remote site enhancing the quality of diagnosis. Other areas where the use of telepathology may grow include continue education, proficiency testing, recertification of pathologists and other laboratory personnel. As the quality and speed of video transmission are improved, the effectiveness of telepathology will be enhanced. It is important as this field expands to secure legislation that would require state medical licensure for out-of-state pathologists who practice medicine.

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ALTERNATIVE METHODOLOGY USING DIGITAL IMAGE ANALYSIS AND ARTIFICIAL NEURAL NETWORKS TO ANALYZE THE FOLLICULAR LYMPHOID DEPLETION IN THE BURSA OF FABRICIUS

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INTRODUCTION

The bursa of Fabricius (BF) is involved in many immunosuppressive diseases and has an important role in the characterization and diagnosis as well as in the monitoring of such conditions. Currently, the evaluation of bursal lymphoid depletion depends on a subjective histological evaluation therefore susceptible to errors. In fact, a lesion score has been reported in order to minimize possible subjective errors (6,7,10).

Image analysis (IA) is a recent tool used in many scientific areas, such as food quality (3,4), agriculture (8,19), and as an aid in disease diagnosis (1,18). The ANN has been widely used in many scientific areas but presently is under-utilized in the poultry industry and has been scarcely reported in the literature. Salle *et al*.

(15) proposed its use for breeders management, in the broiler breeders serological results interpretation (14), in the management of broiler flocks (16), hatchery management (17) and as an aid for evaluation of *Escherichia coli* pathogenicity (11). The ANN was used further to identify the propensity of chickens to develop pulmonary hypertension syndrome (12), to evaluate the minimal invasive measurements for early diagnosis of ascites in broilers (13) and to detect wholesome or unwholesome carcasses in on-line inspection (2).

In this paper we report the use of IA and ANN techniques to minimize errors that may occur in the conventional optical technology.

MATERIALS AND METHODS

Bursa of Fabricius samples. Fifty BF samples were collected intact and kept in individuals vials with 10% buffered formalin. These organs were collected in the previous experiment realized by Moraes *et al*. (7).

Histological examination. All BF samples were processed for histological examination following the standard technique (5) and cut at the level of their major diameter in order to obtain the largest observation area.

The slides were first evaluated throughout the conventional subjective method (optical), and the degree of lymphoid depletion scored in a scale from 1 to 5: Score 1 = < 25% depletion; Score 2 = 25 to 50% depletion; Score 3 = 50 to 75% depletion; Score 4 = 75 to 90% depletion; and Score 5 = >90% depletion (9). The samples were scored three times by a single examiner with an interval of one day between examinations.

Image capture. Ten slides of each score were randomly selected for image processing. Each slide was divided in four parts, and three follicles for each quadrant were selected for examination. The quadrants were numbered clock-wise (I to IV) and the follicles in crescent order (1 to 12).

Finally digital photomicrographic images were taken under a 20X magnification with an OLYMPUS® C-7070 camera.

Image analysis. The images were assayed using the Matlab® 6.5 software according to the following steps: (a) change to gray scale; (b) select the follicular area; (c) all structures around the selected area were rubbed out; (d) the number of pixels for gray scale of each image was estimated; and (e) the image histograms and table were constructed.

Areas of the histogram were selected and their corresponding data (right and left area, area at the mid-point, midpoint values at X (major index) and Y (higher point) provide the input to construct the ANN. Identification and digital classification of the follicles with different lymphoid depletion scores was performed using a commercial ANN software. This data was used to train the ANN to obtain a digital score which is compared with the score obtained by optical classification. This procedure is performed for each selected follicle.

RESULTS

The ANN was able to make a comparable classification of digital and optical scores. High sensibility and specificity was found in scores 1 where only nine follicles were misclassified as score 2. It was more difficult to find agreement between ANN and optical classification in more severe cases of depletion and both sensibility and specificity decreased in scores 3, 4 and 5 (Table 1).

In attempt to improve accuracy of the ANN, scores 1 and 2, 4 and 5, were agglutinated and named 1A and 5A, respectively. Score 3 was maintained intact. In spite of this change the ANN performance remained similar to previous classification, having a difficult of classification in score 3, where 31 follicles were misclassified as 5A. The sensibility and the specificity were respectively 79.39% and 91.94% for score 1A, 62.5% and 79.27% for score 3; and 65.42% and 85.92% for score 5A.

Aiming to improve accuracy of the ANN the follicles were then grouped in a binary fashion, separating the scores of the BF in normal and clearly abnormal. Scores 1 and 2, and scores 3, 4 and 5 were agglutinated in score 1B and 5B, respectively. With this alternative the accuracy of the ANN improved significantly with sensibility and specificity of 92.54% and 90.28% for the score 1B, and 90.28% and 92.54% for score 5B, respectively.

DISCUSSION

The results of this study suggest that the digital image analysis using the Matlab® 6.5 combined with the ANN is a helpful tool in the diagnosis of follicular lymphoid depletion in BF. One advantage of this new methodology is that it does not need special histological techniques, since it can be performed with routine H&E staining. In addition it does not require a sophisticated training to implement the referred protocol. The use of digital imaging reduces the subjectivity of the conventional optical technique and potentially improves the depletion accuracy.

The use of ANN showed to be efficient for all digital score lesions with less accuracy in scores 2, 3, and 4. This inconsistency maybe due to the fact that the images for these scores could have similar appearance on routine optical evaluation and is encountered even

when experienced pathologists perform their histological assessment.

The optical score 1 and 2 are similar histologically, and often pose difficulties for the pathologist to allocate in one or in the other score. The same difficulty is encountered when ANN methodology is used, but with the advantaged that the "error" (sensibility and specificity) is informed by the software. This difficulty is also seen for the follicles with optical score 3, since only 53 samples (44.17%) were correctly evaluated and 43 (35.83%) were allocated on scores 4 and 5. A similar confounding area is also detected for the optical scores 4 and 5.

The ANN performance improves with grouping of the scores. When the scores are grouped in a binary fashion, simulating a more realistic situation where the animal is either healthy or sick, the ANN accuracy increases significantly. This is reinforced by Pereira (10) and Moraes *et al*. (7) who suggest that a bird should be considered sick only when BF using optical score is 3 or more. Therefore regrouping the scores in a binary fashion for ANN training does make sense.

The use of ANN to determine the pathogenicity index (PI) for *Escherichia coli* has been recently reported by Rocha (11). The author classified the PI in 10 categories and found a variable sensibility but a high specificity (above 95%). Similar to our study, the variability in sensibility was attributed to the number of the PI categories. When the PI was classified in three categories: non virulent, of intermediary pathogenicity and virulent the ANN gave more reliable results.

The results found in this study suggest that the use of digital image analysis and ANN is a helpful tool for routine laboratorial evaluation of BF histological lesions. The use of ANN allows a more accurate and reliable reading of the histological lymphoid depletion, independent of the operator or laboratory where it is carried out. As the ANN constructs an objective scale the results can be compared from different laboratories and therefore is advantageous when compared to the conventional subjective methodology. For instance, poultry companies with subsidiaries in distinct geographic areas could exchange data related to monitoring programs and elaborate a realistic risk analysis for BF lymphoid depletion.

Finally, it must be emphasized that although the use of ANN enhances the ability to evaluate BF lymphoid depletion, the presence of a trained pathologist is essential for the differential diagnosis of other conditions.

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Table 1. Classification of follicles using digital scores and conventional optical scores.

Scores	OS1	OS 2	OS 3	OS 4	OS 5	Total
DS1	**97**	26	19	1	3	146
DS2	9	**67**	5	0	4	85
DS3	1	10	**53**	20	34	118
DS4	0	0	11	**84**	17	112
DS5	1	17	32	15	**62**	127
Total	108	120	120	120	120	588
Sensibility (%)	89.81	55.83	44.17	70.00	51.67	
Specificity (%)	89.79	96.17	86.11	94.02	88.11	

OS = optical score; DS = digital score.

BIOCHEMICAL BEHAVIOR OF *ESCHERICHIA COLI* ISOLATES FROM BROILERS THROUGH THE USE OF ARTIFICIAL NEURAL NETWORKS

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INTRODUCTION

Works with *E. coli*, in which artificial neural networks were used, essentially approach the basis of genetic identification of DNA promoters (10,6), bacterial growth (4,5), DNA sequence (6), mutagenicity, hepatotoxicity and teratogenicity prediction (9), among others.

In the poultry industry, the first models that used networks were published by Zhang *et al*. in 1996 (22), in the nutrition field. In relation to broilers health, Brazilian researchers have already proposed the use of mathematical models based on conventional statistics, which explained the immune response of breeders (16) and used artificial neural networks in this industry (17,18,11,19).

Escherichia coli is a microorganism known for causing diseases in broilers, although it is not clear yet if it is a primary or secondary cause for the diseases attributed to it (8). Ferreira & Köbl (2000) (2) stated that *E. coli* is considered to be secondary to other agents, being the cause of extra-intestinal diseases in broilers. We find many lesions in broilers contaminated with this bacterium, such as colisepticemia, airsacculitis, peritonitis, pleuropneumonia, pneumonia, swollen head syndrome, osteomyelitis, among others (1,2,8).

According to Rocha (1999) (12), the most frequent virulence factors detected in respiratory isolates of *E. coli*, isolated in Rio Grande do Sul, were serum resistance and colicin production.

Virulence mechanisms of *E. coli* isolates potentially pathogenic for broilers (APEC) have been continuously studied and it is supposed to be of multifactorial cause. Certain properties are primarily associated with these strains and the most frequently mentioned include adhesion ability (*pap* and *fel*), production of colicins (*cva*), presence of aerobactin (*iut*), serum resistance (*iss*) (Barnes, 1994) (1), temperature sensitive hemagglutinin (*tsh*), and presence of some capsular antigens (kps) (7).

Improvements in research and tools used have resulted in a greater understanding of the mechanisms of pathogenicity, and it is increasingly being proved the importance of the interaction of different virulence factors in determining the pathogenicity. However, what sets apart virulent and avirulent strains remains a problem in diagnosis and, consequently, in the decision-making by the field veterinarian. There is no standardization of procedures and classifications in determining the *in vivo* pathogenicity index (PI), which prevents the comparison of results. In an attempt to solve these problems, Souza, (2006) (21) proposes an objective classification based on numerical results, which makes possible the ananlysis of data using statistical ananlysis and allows the comparison of results. In this classification, the pathogenicity index ranges from 0 to 10 (10).

Isolated PCR tests (uniplex) were used to identify seven genes associated with virulence of *E. coli* (13,14). Later, Rocha (2008) (15) developed PCR multiplex by working with the same isolates from 2006.

Antimicrobial resistance is a major concern for public health. In 2011 (20), Salle *et al*. characterized the resistance or susceptibility of isolates of *Escherichia coli*, which were used for the paper, in comparison with 14 antimicrobial agents using artificial neural networks. These results will be used for the studies proposed here.

Recently, Fortes (2008) (3) studied the biochemical behavior of 261 *E. coli* isolates from broilers. The results showed that the positive isolates for arginine, dulcitol, raffinose, and sucrose have higher pathogenicity index (PI) than the others. On the other hand, negative isolates for salicin and for indole test also have PI higher than the positive isolates. He also concluded that salicin, sucrose, raffinose, adonitol, and dulcitol as well as arginine and ornithine, showed varying results for *E. coli*.

The objective of this paper is to build artificial neural networks to predict the results of the variable biochemical tests reported by Fortes (2008) (3). As inputs, we used existing information at the Center of Diagnosis and Research in Avian Pathology (CDPA) database.

MATERIALS AND METHODS

The data used in this paper is gathered in a database at the CDPA, Veterinary Faculty, Federal University of Rio Grande do Sul, Brazil. We used the following information on 261 *Escherichia coli* isolates:

- biochemical characteristics (arginine, dulcitol, ornithine, raffinose, sucrose, indol, and salicin);
- pathogenicity indices (0 to 10);
- lesions caused by inoculation of one-day-old chicks (cellulitis, peritonitis, perihepatitis, pericarditis, and airsacculitis);
- characterization of genes associated with pathogenicity (*papC, felA, cvaC, iutA, ISS, tsh*, and *kpsII)*
- antimicrobial resistance to 14 antibiotics (amikacin, amoxicillin/clavulanic acid, ampicillin, cephalexin, ceftiofur, cefuroxime, ciprofloxacin, clindamycin, cotrimoxazole, enrofloxacin, gentamicin, norfloxacin, ofloxacin, and tetracycline);
- origin of isolates (broiler litter, cellulitis, and respiratory pictures); and
- bacterial motility.

The artificial neural networks were built through the use of Neuroshell Classifier 2.1 software (Ward Systems Group, Inc., Frederick, MD, USA, 1997-2000).

The results were converted to binary format. Thus, negative results are represented by number 0 and positive results by 1. This criterion was used for sensitivity (0) or resistance (1) to the 14 antibiotics studied, for absence (0) or presence (1) of genes associated with pathogenicity, for absence (0) or presence (1) of different lesions observed in chicks. Non-motile isolates were represented by 0 and motile isolates by 1. For the case of isolates origin, it was necessary to add another value as follows: litter (0), cellulitis (1) and respiratory pictures (2)

Inputs chosen to build the artificial neural networks were pathogenicity indices, lesions induced in one-day-old chicks, characterization of genes associated with pathogenicity, antimicrobial resistance, origin of isolates, and bacterial motility. Outputs chosen were variable biochemical reactions obtained by Fortes (2008) (6): Ornithine, arginine, adonitol, raffinose, sucrose, salicin, and dulcitol.

RESULTS AND DISCUSSION

Table 1 shows the characteristics of the artificial neural networks built with following inputs: antimicrobial resistance (Atb), genes associated with pathogenicity (G), pathogenicity index (PI), motility of *E. coli* (M), lesions produced in one-day-old chicks (L), and origin of isolates (O). Outputs are the different biochemical reactions classified as variable by Fortes (2008) (6).

Table 1 also shows that the artificial neural networks were able to classify with high accuracy the positive or negative result of all biochemical tests studied. The worst result, but still very good, was obtained for salicin with 87.80% for correct classification, followed by sensitivity with 88% and specificity with 87.6%. On the other extreme we find arginine with 98.37% for correct classification and, for sensitivity and specificity, the results were 99.47% and 94.64%, respectively. The remaining biochemical tests results are between these two extremes, as shown above, and demonstrate the ability of the neural networks to classify the results of the biochemical tests as from information available.

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Table 1. Classification of biochemical reactions, positive or negative, of 261 *Escherichia coli* isolates, through the use of artificial neural networks.

Output	Inputs	Correct Classification (%)	Sensitivity	Specificity
Adonitol	Atb + G + PI + M + L + O	94.72	0.9895	0.8000
Arginine	Atb + G + PI + M + L	98.37	0.9947	0.9464
Dulcitol	Atb + G + PI + M + L + O	94.31	0.7963	0.9844
Ornithine	Atb + G + PI + M + L	90.24	0.5932	0.9519
Raffinose	Atb + G + PI + M + L	96.34	0.8500	0.9854
Sucrose	Atb + G + PI + M + L + O	90.65	0.8028	0.9486
Salicin	Atb + G + PI + M + L + O	87.80	0.8800	0.876

POTENTIAL ROLE OF *DERMANYSSUS GALLINAE* AS A CARRIER OF *SALMONELLA* GALLINARUM

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SUMMARY

Dermanyssus gallinae has been proved to be a vector for many viral and bacterial pathogens. We aimed a study to assess the potential role of mite as a carrier of *Salmonella enterica* subsp. *enterica* ser. Gallinarum (*S.* Gallinarum), a pathogen that causes great concern for its heavy detrimental effects on poultry.

We collected 45 samples of mites from as many Italian poultry farms, and we used a specific and sensitive seminested PCR system to assess the possible

presence of *S*. Gallinarum among them. Three samples resulted positive. To verify if *S.* Gallinarum might be present on the surface or inside the mites, we washed the positive samples to remove the potential outer contamination before repeating the seminested PCRs. The samples returned positive results again.

Our findings are consistent with a potential vectorial role of *D. gallinae* for *S*. Gallinarum.

INTRODUCTION

The red poultry mite, *Dermanyssus gallinae*, is one of the most widespread ectoparasite in the poultry industry, as its prevalence may reach 90% of poultry farms (5). In Italy, an average infestation rate of 74.1% has been estimated on laying hen farms. The economical impact of the infestation is relevant, as *D. gallinae* may lead to decreased production and poor bird welfare. Furthermore, a number of studies have suggested a role of *D. gallinae* as a vector for animal and human pathogens, such as viruses, bacteria, and parasites (2,6). However, it is not always possible to establish whether the mite plays an active role as a vector or whether it is only a carrier of infectious agents (6).

Those considerations led us to investigate about the potential role of *D. gallinae* as a vector of *S.* Gallinarum. The pathogen is considered a major threat for chicken flocks since it is the causative agent of fowl typhoid, currently one of the most important causes of poultry mortality and economic losses in many countries (4).

MATERIALS AND METHODS

D. gallinae were collected from 45 naturally infested laying and breeder farms from Italy.

The mites were collected by farmers following our instructions, and they were sent anonymously to the facilities of the Avian Pathology Unit of the Dept. of Public Health and Animal Sciences of University of Bari. We have not retrieved any information about potential history of fowl typhoid in the poultry farms.

Mites were identified as *D. gallinae* according to the Varma's and Baker's morphological keys (1,8).

Groups of 100 mites from each sample were homogenized and total DNA extraction was carried out by using the TRI Reagent (Sigma, Milan, Italy) according to the manufacturer's instructions. DNA extraction was performed directly from the homogenates.

Four µL of DNA solution were used as template in the *S.* Gallinarum specific seminested PCR according to Pugliese *et al*. (3). Amplification products of each step of seminested PCRs were analyzed by electrophoresis through 1.5% agarose gel and visualized after staining with ethidium bromide.

Two further aliquots of 100 mites from PCR positive samples were collected and washed three times with 75% ethanol before homogenization and total DNA extraction. DNA from washed mites was used as template for *S.* Gallinarum specific seminested PCRs.

RESULTS AND DISCUSSION

Three out of the 45 samples (6.7%) resulted positive to both the seminested PCRs. This finding highlights the persistent circulation of *S.* Gallinarum among the Italian poultry farms and it reveals the suitability of the PCR-based approach in epidemiological investigations.

On the other hand, the result discloses association between *S.* Gallinarum and *D. gallinae*.

To assess if the pathogen laid on the surface of mites or if it were harbored inside *D. gallinae*, we washed two aliquots of mites from the positive samples before repeating DNA extraction and the seminested PCRs. Also in this case, PCRs returned positive results.

Those findings confirmed the internal presence of *S.* Gallinarum in mites, hence strengthening the hypothesis that *D. gallinae* may act as a vector for the poultry pathogen.

Some authors reported that *Salmonella enterica* subsp. *enterica* ser. Enteritidis replicates itself inside *D. gallinae* (6,7). If those findings were also confirmed for *S.* Gallinarum, it would be suggested a potential role of *D. gallinae* as a reservoir for the infection. Not recently, some authors stated that *D. gallinae* could play a role in the diffusion of fowl typhoid, as infected mites surviving after an outbreak could spread the infection during their subsequent production cycle (9). However the matter remains controversial, and questions are raised about the active or passive role of *D. gallinae* in the diffusion and recrudescence of fowl typhoid.

We are directing our efforts to ascertain how *S.* Gallinarum infects the red mite and if *D. gallinae* may transmit the infection.

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EFFECTS OF IMMUNOSUPRESSION ON THE DEVELOPMENT OF CELLULITIS IN TURKEYS

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Over the last few years, cellulitis/clostridial dermatitis has become more prevalent in commercial turkeys in Minnesota and elsewhere. Cellulitis is described in turkeys as a condition characterized by inflammation of the skin and subcutaneous tissue with accumulation of focal yellow or yellow-brown exudate in the subcutis of breast and tail areas. Accompanied by high mortality and carcass condemnation in the processing plants, cellulitis often inflicts heavy economic loss for the turkey producers.

Most often, the bacterial agents isolated belong to *Clostridium perfringens* and *Clostridium septicum* group of organisms. *Clostridium perfringens* and *C. septicum* are both spore forming bacteria and their spores are highly stable and ubiquitously present in the environment. These pathogens are capable of producing a myriad of extracellular toxins and enzymes that degrade host tissues and are responsible for the necrotic lesions observed. However, there is very little information as to the exact and to what extent these clostridial organisms play a role in clostridial dermatitis in turkeys or does immunosuppression also play a role in the development of cellulitis.

Birds experience lots of stress, especially in the intensive system of rearing. A stress can suppress the immune system in a healthy bird and predispose it to clostridial infections. Oral challenge with clostridial spores was not sufficient to develop cellulitis in experimental turkey poults in our earlier studies.

Our objective of this study was to examine the role of *C. perfringens* and *C. septicum* for clostridial dermatitis in causing cellulitis in turkeys in an immunosuppressive background. We immunesuppressed nine-week old turkey poults with a chemical, dexamethasone, intramuscularly. The birds that were immunosuppressed and not immunesuppressed were then challenged with *C. perfringens* and/or *C. septicum* orally to examine the development of clostridial dermatitis. The dexamethasone treated birds were found to be more susceptible to *C. perfringens*/C. septicum challenge and developed cellulitis than the non-dexamethasone treated birds.

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

CHANGES IN THE SECRETORY PROTEIN PROFILES OF *CLOSTRIDIUM PERFRINGENS* FROM CELLULITIS CASES IN TURKEYS

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Turkey cellulitis is an acute infectious disease, and has caused significant economic losses in the US turkey industry in many parts of USA, which included Minnesota and Wisconsin and other places (1). *Clostridium perfringens* has been consistently isolated from cases of cellulitis in turkeys apart from *Clostridium septicum* (2). These belong to a group of anaerobic organisms affecting animals and they are described as histotoxic clostridia. They are most commonly involved in gas gangrene, or myonecrosis secondary to wound infections. They include *C. perfringens*, *C. septicum*, *C. novyi*, and *C. bifermentans* (3). *Clostridium perfringens* is one of the most widespread of all pathogenic bacteria affecting humans and animals. Its main habitats are the soil and the intestinal tracts of animals and man. Armed with an arsenal of many potent extra-cellular toxins and enzymes, *C. perfringens* is recognized as the causative agent of human gas gangrene and food poisoning as well as several enterotoxemic diseases in livestock (4).

Clostridium perfringens is found in the intestinal tract of healthy poultry as a normal inhabitant, usually in low numbers and has been isolated from processed carcasses as well as from the processing plants (5,6). Hatcheries are identified as a potential source and reservoir for *C. perfringens* in integrated poultry operations (5,7). Experimental inoculation of purified alpha toxin of *C. perfringens* produced lesions similar to cellulitis and also severe mortality in turkeys (7).

Necrotic enteritis and gangrenous dermatitis are among the most common clostridial diseases observed in broiler chickens which are caused by *Clostridium perfringens*. In turkeys, clostridial cellulitis which is similar to gangrenous dermatitis in chickens is the major disease caused by *C. perfringens* type A and *C. septicum*. It is interesting to note that though both necrotic enteritis and gangrenous dermatitis in broilers are attributed to *C. perfringens* type A, both disease conditions are not yet reported in any flocks or in any farms at the same time (8). Moreover, the incidence of one or the other disease condition continues to be more pronounced in some well maintained farms than in others in spite of adopting strict control strategies. It appears that the *C. perfringens* responsible for each disease conditions are different.

Clostridium perfringens isolates are grouped into types A through E based on their production of one of the four major lethal toxins; alpha, beta, epsilon, and iota (9). The growth of *C. perfringens* is restricted to the site of infection, whereas the alpha toxin produced is absorbed by circulatory system and causes massive intravascular hemolysis and destruction of capillary walls. All five types of *C. perfringens* produces alpha toxin which is an enzyme phospholipase C also called as lecithinase (10). In addition, *C. perfringens* produces an array of extracellular toxins including beta2 toxin, enterotoxin, perfringolysin, collagenase, lambda toxin, hyaluronidase, DNase, neuraminidase, and urease (9). The sporulation process in *C. perfringens* is linked with the toxin production (11).

Recently many hypothetical proteases and β2-toxins were found in virulent *C. perfringens* type A strains and that is believed to play a major role in eliciting a protective immune response against necrotic enteritis. A novel toxin NetB is now identified as a definitive virulence factor present in avian *C. perfringens* strains capable of causing necrotic enteritis in chickens (12). Another group of scientists recently demonstrated a hypothetical protein of 117 kDa suggestive of a protease found only in virulent *C. perfringens* type A causing necrotic enteritis. This protein is believed to play a major role in the development of necrotic enteritis and also in eliciting a protective immune response (13). It is also shown that the β2-toxin gene (*cpb2*) found in isolates of *C. perfringens* cultured from avian hosts are atypical compared to those found in pigs (14). It is not clear whether the above mentioned proteins play any role in the pathology of cellulitis in turkeys.

In this study, we examined the secretory components of *Clostridium perfringens* that causes cellulitis in turkeys. In brief, *C. perfringens* type A isolates that produced cellulitis and those which did not produce cellulitis were included in this study. They were grown in sporulation media and allowed to express toxins. The culture supernatant from these isolates was subjected to SDS-PAGE analysis and two-dimensional gel electrophoresis to separate the proteins. A western blot was performed using convalescent sera from the birds exposed and non-

exposed to *C. perfringens* toxins. The reactive toxin components were identified by MALDI-TOF mass spectrometry.

We observed expression of many toxins in varying quantities besides alpha-toxin which possibly may play a role in causing cellulitis in turkeys. Our results suggest expression of more toxins by *Clostridium perfringens* type A that appear to be potent in causing cellulitis than *Clostridium perfringens* type A, which did not produce cellulitis. The results of this study will be presented.

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CASE STUDY: INFLUENCE OF MINERAL CONTENT AND BIO-MOS® ON CRACKED AND UNDERGRADE EGGS

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I NTRODUCTION

Broilers grown with low levels of Bioplex minerals had comparable performance to those grown with traditional levels of inorganic minerals (1). This study was followed up with a layer study (2) which showed that layer performance could be maintained though the first cycle at levels comparable to that on traditional inorganic trace mineral levels with substantially lower levels when trace mineral were provided in the Bioplex form. This line of research was continued, with effects on shell quality being demonstrated (3,4,5) reported.

Based on this, Dave Coburn, Coburn Farms replaced the inorganic trace minerals in his feed with low levels of Bioplex minerals plus Sel-Plex and iodine. This case study covers the flock prior to this change plus the two flocks subsequent to the change.

MATERIALS AND METHODS

The premix provided supplemental mineral levels using Bioplex and Sel-Plex, of zinc 14.0 ppm, manganese 12.6 ppm, copper 0.6 ppm, iodine 0.8 ppm, and selenium 0.3 ppm in the complete feed. Flock 22 was a mixture of Lohmann LSL and Shaver White Leghorns; flocks 23 and 24 were all Lohmann LSL. All feed was fed as mash and milled on farm. The hammermill used a ½ inch screen to provide a coarse mash (6) and 6% oats was locked into all rations for the three flocks to assure adequate fiber levels (7). The birds were housed in multi bird cages, and all eggs were shipped approximately four hours prior to grading. Flock 22 was fed traditional levels of inorganic supplemental trace minerals; flocks 23 and 24 were fed Bioplex/Sel-Plex minerals at the above levels. In flocks 22 and 23 there was trace amounts of Bio-Mos; in Flock 24 this was raised to 1 kg per tonne. Flocks were depleted at 68 and 69 weeks of age.

RESULTS AND DISCUSSION

Performance was at good levels for all three flocks (Table 1); eggs/HH project to 70 weeks averaged 315.7, 315.7 and 324.1; mortality was 7.7, 7.7 and 4.7%; with weeks over 90% being 29, 35 and 39 for flocks 22, 23 and 24 respectively. The improvement from flock 23 to 24 was particularly noticeable. Bio-Mos was in at 300g/tonne in flock 22 and 24 then early in lay in flock 24 was increased to 1 kg/tonne. This was done as it was speculated that gut health would improve and thus dirty eggs would decrease. Improved gut health would also seem to be reflected in improved livability and thus improved eggs/HH. It should be noted that in flock 24 greater attention was paid to assuring all birds accessed the nipple drinkers after housing in the layer cages.

Cracked eggs for flock 23 with Bioplex/Sel-Plex trace minerals was less than 60% of that seen the previous flock when inorganic minerals were used. Downgrades for flock 23 were just over 60% of the previous flock. It should be noted that these categories of eggs for Flock 22 with inorganic trace minerals were at levels that are in the very acceptable range for the industry. Thus, Flock 23 was at very low levels for the industry. When Bio-Mos was added at 1 kg per tonne in addition to Bioplex/Sel-Plex minerals, for Flock 24, cracks and down grades were further reduced to 0.64 and 1.52% respectively.

Livability, eggs/HH, weeks over 90%, cracked eggs, and total undergrades improved through bird management and the use of Bio-Mos, Bioplex minerals, and Sel-Plex.

ACKNOWLEDGEMET

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Table 1. Flock performance.

	Flock 22 2007-2008	**Flock 23 2008-2009**	**Flock 24 2009-2010**
Birds housed	25,610	25,342	25,400
Mortality, %	7.7		4.7
Birds shipped	23,627	23,381	24,196
Eggs/HH	305.4/68 wks	309.4/69 wks	318.1/69 wks
Eggs/HH projected to 70 wks	315.7	315.7	324.1
Wks over 90%	29	35	39
Wks over 80%	45	48	47
Feed /doz. eggs ,g	1430	1453	1442
Feed /doz. eggs , lbs		3.20	3.17
% large and above	75.56	85.52	86.8
%, medium	15.68	9.6	10.31
%, small	3.24	4.34	1.17
%, pee wee	0.84	0.65	2.04
%, cracks	1.78	1.05	0.64
%, total down grades	4.93	3.04	1.52

NUTRIFIBE™ COMPLEX ALONE OR COMBINED WITH BMD® IMPROVES BROILER PERFORMANCE COMPARED WITH BMD ALONE

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SUMMARY

BMD® is commonly used as a growth promoter in broiler feeds to improve gain and feed efficiency. As producers seek alternatives to support gut health, choices include natural functional ingredients. NutriFibe™ Complex (NFC) is a new product developed to support gut health and immunity. It combines prebiotic fibers, beta glucans and yucca to promote a desired microflora and enhance gut integrity. This study compared broiler performance on diets containing NFC, BMD or NFC + BMD. There were eight floor pens with 45 Cobb male broilers per pen for each treatment. Results on d 35 showed that average weight gain was similar in all treatment groups. Feed efficiency was statistically better in the birds fed NFC alone or NFC + BMD than BMD alone ($P<0.05$). Feed efficiency was 1.8 points higher for the NFC-fed birds and 3.0 points higher for birds fed NFC+BMD. These results confirm field observations and demonstrate that optimizing gut health pays off in performance and feed efficiency.

INTRODUCTION

To meet the nutritional needs of high performance birds, chicks need to develop a healthy, well-functioning gut early in life. A balanced gut microflora can maintain an effective barrier against overgrowth by enteric pathogens like *Clostridium perfringens*. NutriFibe Complex is a new product from Ralco Animal Health that is designed to support gut health and promote the development of a balanced gut microflora. This product is a unique combination of proprietary prebiotic fiber, mannan oligosaccharides, and *Yucca schidigera* extract. The prebiotic fiber is selectively fermented by the beneficial bacteria, *Bifidobacterium* and *Lactobacillus*. As these bacteria

multiply, they produce short chain fatty acids (SCFA) that reduce pH and promote maintenance of the gut epithelial cell barrier. The beta glucans support immune cell function and yucca extract is a source of natural saponins that are antiprotozoal and improve nutrient absorption. The objective of this trial was to determine if NutriFibe Complex alone or combined with BMD would improve broiler performance when compared with BMD alone.

MATERIALS AND METHODS

This study was performed at Southern Poultry Research, Inc. (Athens, GA). There were three treatments: NFC, BMD or NFC+BMD. The three treatments were replicated in eight blocks, with the treatments randomized within each block. The pen was the experimental unit. The floor pen house is a modified poultry house with dirt floors and curtain sidewalls. Each pen had an area of 4 x 10 = 40 ft^2 (3.72 m^2), with clean wood shavings as bedding with thickness of approximately four inches. The stocking density was 0.81 ft^2/bird. The diets were provided *ad libitum* in one tube-type feeder per pen. From d 1 until d 7, feed was also supplied on feeder trays, placed on the litter. Water was provided *ad libitum* from one per pen Plasson-type automatic watering fount. Day of hatch Cobb male chicks were obtained from Cobb-Vantress hatchery, Cleveland, GA with 45 chickens per pen. Dead birds were recorded daily and weighed. Diets contained Maxiban® (90 g/t) and 3-Nitro 20 (45.4 g/t) in all starter and grower feeds. All feeds were fed as crumbles/pellets.

RESULTS

Results (Table 1) showed that on d 19, birds on BMD alone had greater weight gain and improved feed efficiency compared with birds fed NutriFibe Complex alone or NutriFibe Complex + BMD. However, on d 35, weight gain was the same in all groups and feed efficiency was statistically lower for birds fed NutriFibe Complex alone or in combination with BMD. Feed efficiency was 1.8 points higher for the NutriFibe Complex birds and three points higher for birds fed NutriFibe Complex + BMD.

DISCUSSION

Awareness of the importance of gut health is increasing. A healthy gut epithelium allows for optimum nutrient uptake. A healthy and balanced microflora blocks enteric pathogens by competitive exclusion and supports intestinal immunity. Approximately 70% of the body's immune cells and tissues are located in the gut. This "gut associated lymphoid tissue" or GALT is the first line of defense against invading pathogens. The intestinal microflora influence maturation of the GALT shortly after hatch and provide important immune system regulatory signals.

One of the key ingredients in NutriFibe Complex is a proprietary prebiotic fiber which is preferentially metabolized by *Bifidobacterium* and *Lactobacillus.* This fermentation results in increased numbers of beneficial bacteria. *In vivo* studies of of broiler gut flora showed that when chicks were fed this prebiotic fiber over a six week time period, *Lactobacillus* populations increased while *Clostridium* numbers were reduced. During fermentation, short chain fatty acids (SCFA) are produced. Butryic acid is about 30% of the total SCFA produced. Butyric acid provides energy directly to the intestinal epithelial cell layer to maintain optimum nutrient absorption. In addition, SCFA reduce local pH making the environment hostile for many pathogens.

The results of this study showed that by the end of the growing period, NutriFibe Complex supported excellent weight gain and delivered improved feed conversion compared to BMD. When combined with BMD, NutriFibe Complex showed a three point advantage in feed conversion. These results demonstrate that optimizing gut health pays off in performance and feed efficiency.

Table 1. Effects of NutriFibe™ Complex (NFC), BMD® or NutriFibe Complex + BMD on broiler performance.

Trait	Day 19			Day 35		
	BMD	NFC	NFC+BMD	BMD	NFC	NFC+BMD
Ave wt. gain (kg)	0.532	0.506	0.514	1.846	1.859	1.866
Pen feed disappearance (kg)	36.04	35.49	35.72	127.24	125.39	124.74
Feed:gain	1.414	1.469	1.445	1.554	1.536	1.524
Death loss (%)	1.7	2.3	2.0	5.0	7.1	6.5

EFFICACY OF VACCINATION PROGRAMS BASED ON A HVT/ND-RECOMBINANT VACCINE AGAINST NEWCASTLE DISEASE AS AN ALTERNATIVE TO CONVENTIONAL PROGRAMS

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SUMMARY

The use of innovative biotechnology techniques has contributed to the development of a new generation of so called recombinant vaccines. Recombinant vaccines show characteristics that can replace or complement the use of conventional live or inactivated vaccines, and therefore contribute to the improvement of control programs against Newcastle disease (ND) in the poultry industry. The use of a recombinant vaccine against Marek's disease (MD) and ND in a turkey herpes virus (rHVT ND SB) vector is discussed with examples that demonstrate its efficacy in circumstances that mimic low and high ND challenge areas. Single vaccination with rHVT ND SB resulted in early and complete protection in commercial (MDA+) broilers against challenge with the velogenic Texas GB strain at 15 d of age. This offers an alternative for early ND protection in areas under low ND field pressure, without the use of additional field boosting. Early onset of immunity is critical for severe ND challenge areas. Therefore, for areas with a high ND field pressure the rHVT ND SB vaccine can be used in combination with a hatchery vaccination using a live attenuated ND vaccine. As an example, the use of rHVT ND SB + live attenuated ND vaccine strain Clone 30 resulted in 100% protection after challenge with the velogenic Mexican Chimalhuacán ND strain from day 11 onward in commercial (MDA+) broilers

INTRODUCTION

Newcastle disease (ND) is an acute viral infection of poultry of all ages that in severe and uncontrolled situations can result in high mortality and significant economic losses. The impact of infection will depend on the virulence of the virus strains present in the field and the prevention measures in place. Vaccination programs against ND differ from country to country or even from area to area within a country because of the diversity in field challenge. Achieving early protection against ND is of utmost importance no matter the vaccination program used or the level of infection pressure in the field.

Protection against ND is critical in countries with velogenic ND challenge. In those areas velogenic NDV field isolates commonly have a high intracerebral pathogenicity index (ICPI) of 1.80 to 1.94 out of a maximum total of 2.0. To achieve protection in this high challenge environment, broiler breeders are hyperimmunized against ND to induce high maternal antibodies, and then the broiler vaccination programs employ multiple live and inactivated ND vaccines. Sometimes a combination of live and inactivated ND vaccine is already administered at the hatchery.

The use of conventional live attenuated ND vaccines during the first days of life will result in early protection against ND challenge. Nevertheless, in areas of low infection pressure, vaccination of young chicks with live vaccines is often considered unnecessary and the cause of undesired respiratory reactions. Complications with secondary bacterial infections may occur causing stress in the birds, impair performance, and may require antibiotic treatment increasing production costs. Under such circumstances, the use of a recombinant HVT/ND vaccine against MD and ND can be a new possibility for the protection of chickens, as it eliminates the need for live ND vaccination.

Vaccines based on the herpesvirus of turkeys (HVT) have already been extensively used for the prevention of MD. Here, the use of a commercially available, recombinant HVT vaccine developed to help prevent MD as well as ND is described. The vaccine expresses the protective fusionprotein gen of ND virus. To enhance protection against MD the recombinant vaccine is also available in combination with the serotype 2 MD vaccine strain SB1 (rHVT ND SB). This recombinant vaccine offers new possibilities for protection against ND without the drawbacks of current vaccines.

In this paper two experiments are reported. The aim of the first two experiments was to simulate the situation of broilers located in a low ND pressure area - when only one mild live vaccine or no vaccination against ND is used – or in a high ND pressure area – when multiple live and/or inactivated vaccines are used. The onset of immunity and the efficacy of different vaccination protocols including the rHVT-

ND-SB vaccine in protecting commercial broilers (MDA+) against ND challenge were evaluated.

MATERIALS AND METHODS

Experiment 1: Evaluation of protection for low ND challenge areas.

Commercial broiler chicks (MDA+) from hyperimmunized breeders were divided into the following groups:

- rHVT-ND-SB in-ovo vaccinated
- Non-vaccinated MDA+ controls

The level of maternally derived antibodies was measured in siblings by means of a commercial ELISA (IDEXX Laboratories Inc., Westbrook, Maine, USA) according to the manufacturer's recommendations; the average day-old ND geometric mean titer was 3523 (CV 55.3%). Eighteen-day MDA+ embryos were vaccinated with rHVT-ND-SB via *in ovo* injection; no other vaccines were administered to the birds. An unvaccinated group served as control. The groups were challenged at 15 d of age with $10^{5.0}EID_{50}$/bird with the velogenic Texas GB reference strain by eye drop. Birds were observed for 14 d post-challenge for mortality and clinical signs attributable to ND. Birds showing ND signs with lesions or mortality were considered "positive" or not protected. Efficacy of the vaccine was evaluated based upon the percentage of birds that were protected against ND challenge.

Experiment 2: Evaluation of protection for high ND challenge areas.

Commercial broiler chicks (MDA+) from hyperimmunized breeders were used. The level of maternally derived antibodies was measured in siblings by means of the hemagglutination inhibition (HI) test and a commercial ELISA; the average day-old ND geometric mean titer was 512 in the HI test and 7696 in the ELISA test. The chicks were divided into the following groups (10 chicks/group):

- rHVT-ND-SB + ND C2 vaccinated
- rHVT-ND-SB + ND Clone 30 vaccinated
- Non-vaccinated broiler controls (maternal antibody only)
- SPF controls (no maternal antibody)

Test broilers were vaccinated with rHVT-ND-SB via subcutaneous injection (0.2 mL/chick) and then a live ND vaccine (either C2 or Clone 30 strain) was administered via eye drop to match the expected field vaccination program.

Birds from each group were challenged by eye-drop with $10^{6.0}$ EID_{50}/bird of the velogenic Mexican Chimalhuacán ND reference strain at d 11, 19, 28, and 41 of age and were observed for 14 d post-challenge for mortality and clinical signs attributable to ND. Birds showing ND signs with lesions or mortality were considered "positive" or not protected.

RESULTS

Experiment 1. All birds in the rHVT-ND-SB vaccinated group showed complete protection after eye drop challenge at 15 d of age with the velogenic Texas GB reference strain, whereas in the unvaccinated control group 50% of the birds showed mortality and signs of ND (Table 1).

Experiment 2. The high maternal antibodies level provided partial protection at the challenge carried out at 11 d of age (50%) and the protection level declined with age. The rHVT-ND-SB with Nobilis ND C2 live vaccine induced substantial protection (70%) by 11 d of age, and full protection (100%) at 19 d of age or older. When Nobilis ND Clone 30 was applied at one d of age in addition to the rHVT-ND-SB vaccine, protection was already 100% by 11 d of age (Table 1).

DISCUSSION

The rHVT-ND-SB vaccine against MD and ND offers an alternative to traditional programs in both low and high ND field challenge. Single vaccination with the rHVT-ND-SB vaccine offers a suitable alternative for early ND protection in areas under low ND field pressure, without the use of additional field boosting.

In regions with velogenic ND challenge, the current vaccination program involves both live and inactivated ND vaccines. These vaccines are given at the hatchery and again as a field boost. When challenge is severe and occurs in the first two weeks of age, hatchery vaccination with a live attenuated ND vaccine is recommended to maximize early protection. As demonstrated in these trials, full early protection can be achieved without the use of either inactivated vaccine or additional field boosting for velogenic ND challenge conditions.

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Table 1. Percentage of clinical protection after NDV challenge.

Challenge with Texas GB strain at 15 d of age		
Group↓	Number of positive broilers / total after challenge	% of protection
rHVT ND SB In-ovo Vaccinated	0/13	100
Unvaccinated Control Broilers	6/12	50

Challenge with Chimalhuacan strain at "X" d of age				
	11 d	19 d	28 d	41 d
Group↓				
rHVT ND SB s.c.+ live C2	70	100	100	100
rHVT ND SB s.c.+ live Clone 30	100	100	100	100
Unvaccinated Control Broilers	50	20	10	0
Unvaccinated Control SPF's	0	0	0	not done

ANTIBODY RESPONSES OF BROILER BREEDERS AND COMMERCIAL LAYERS POST *SALMONELLA* VACCINATION AND/OR CHALLENGE AS MEASURED BY ELISA

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INTRODUCTION

Salmonella isolations are common from poultry due to prevalence, large number of birds produced, and because of active surveillance programs involving flocks and poultry products in many parts of the world. The global nature of the poultry industry has no doubt contributed to opportunities for the dissemination of *Salmonella*.

Vaccination as a means for limiting *Salmonella* infections in poultry that may have human health significance is widely utilized in both broiler breeders and commercial egg laying chickens. The vaccines utilized include several modified live products that provide local gut associated immunity and inactivated vaccines (bacterins) that induce a more robust systemic antibody response. The inactivated vaccines are supplied as fully licensed products typically containing *Salmonella* Enteritidis or autogenous vaccines that frequently contain multiple serogroups (*Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Kentucky, etc.). Assessing antibody responses pre and post vaccination is important because it can be utilized to monitor vaccine delivery, duration of immunity, field exposure prior to and post vaccination and may have value in determining relative vaccine efficacy when used in combination with other qualitative evaluations (including culture, PCR and challenge).

The purpose of this report is to describe the use of antibody based methodologies (ELISA) to asses antibody responses prior to and post vaccination with live and/or inactivated *Salmonella* vaccines.

PROCEDURES

Commercial layers and broiler breeders were vaccinated with different live and inactivated *Salmonella* vaccines prior to onset of egg production. Sera were collected at several intervals post vaccination and assayed for *Salmonella* antibodies using a *Salmonella* Enteritidis/*Salmonella* Typhimurium ELISA (BioChek B.V. Burg. Bracklaan 57, 2811 BP Reeuwijk, The Netherlands). Onset and duration of antibody responses were assessed up to approximately 48 weeks. Field challenge was monitored by cloacal swabs (PCR and culture).

RESULTS AND CONCLUSIONS

1. The ELISA could be utilized to monitor antibody responses post vaccination with inactivated *Salmonella* vaccines.
2. The ELISA was capable of detecting antibody responses resulting from a field challenge in vaccinated and unvaccinated chicks with either *Salmonella* Group B or *Salmonella* Group D.

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EVOLUTION OF COCCIDIA VACCINES

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Coccidia are hardy ubiquitous organisms but susceptible to desiccation and in a susceptible host could cause considerable damage leading to weight loss, impaired feed utilization, poor pigmentation, and mortality. Nine *Eimeria* species are described for chickens and seven for turkeys. Effective control can be achieved by the use of vaccines and or pharmaceutics.

Drs. Beach (California Experimental Station), Johnson (Oregon Agricultural College), and Tyzzer (Harvard University) in the late 1920s and early 1930s were some of the pioneers in the concept of protecting the host against the disease via providing the host with small doses of sporulated oocysts. Johnson and Tyzzer believed that growing chickens in the absence of all coccidial infections are ill advised. They believed that gradual light exposure to coccidial infections leads to immunity.

As a graduate student S. Allen Edgar (Kansas State U) visited Tyzzer's lab prior to joining U. Wisconsin, where he honed his skills in as a parasitologist. Dr. Edgar later joined the faculty at Alabama Experimental Station (AU) prior to WWII and began the practical approach of non-chemotherapeutics control for coccidiosis as suggested by Beach, Johnson, and Tyzzer. But WWII started and Edgar was deployed to Tahiti where he worked on filarial worms. Following WWII, he continued his pursuit in the development of a vaccine to control coccidiosis. The first coccidial vaccine was developed by Edgar; this product was a live, non-attenuated selected isolate *Eimeria tenella.* This product was marketed in 1952 by Dorn & Mitchell, Inc. The trade names used indicated addition or modifications of the formulations containing various species of highly selected Eimeria strains. The first name was DM® Cecal Coccidiosis Vaccine followed by Coxine® and CocciVac®. *Eimeria mivati* was added to the formulation in mid 1960s. During Dr. Edgar tenure at Auburn U, he also developed the first turkey coccidiosis vaccine. There are two chicken and one turkey formulations of Coccivac. These products had some early acceptance worldwide, but later met some resistance in usage following the discovery of effective anticoccidials.

In the mid 1980s the second coccidial vaccine was developed and marketed by Dr. Eng Hong Lee of Vetech Laboratories, Inc., Canada. There are two formulations for chicken and one for turkeys "Immucox®". Dr. Jeffers of Hess & Clark, Inc. reported his findings on precocious lines of coccidia; this lead to the development of the first attenuated coccidial vaccine by Dr. Shirley and company at the Houghton Poultry Research Station, UK. Glaxo Animal Health Ltd was the owner of the parent, Pitman-Moore, Inc., acquired the rights and the first precocious coccidiosis vaccine Paracox® was launched in 1989. Dr. Long, Houghton Poultry Research Station, UK attenuated coccidia via embryo adaptation in early 1970s. This lead to development of an embryo adapted line of *E. tenella* by Dr Bedrník of the Czech Republic. Livacox® was developed and launched in 1992 by Biopharm,

which includes precocious lines of other species plus the embryo adapted line of *E. tenella*. These four live coccidial vaccines served as foundation products for the development of live non-attenuated, attenuated and precocious products to be launched worldwide.

With the boom of molecular science during late 1970s to 1990s numerous patents for recombinant (subunits, monoclonals etc.) coccidiosis vaccines were submitted; many patents were granted, but to date only one (CoxAbic) has commercial application. Recently, the acceptance of coccidiosis control via non-pharmaceutical means has been gaining momentum for several reasons. This interest has encouraged a boom in development of new coccidiosis products worldwide. There are currently in excess of 15 coccidiosis vaccines (non-attenuated, attenuated, subunit or extracts). The coccidial vaccines can also be characterized as live or killed. The live vaccines confer active immunity and have to be ingested; these products are used in broilers and replacement birds. The killed products are given intra-muscularly (CoxAbic) or orally for IGY (an immunoglobulin) these products confer passive immunity and are used in replacement birds.

During the early years of coccidiosis vaccine usage, the product was applied in the drinking water within the first week of life. As the knowledge base grew about bird behavior and management of the host the products were applied as a spray on to the feed within the first week of life. There were challenges with these methods of applications. In time the administration of the vaccine was removed as a field application to an application in the hatchery where the product was applied via intra-nasal and intra-ocular routes. An edible gelatin puck was also used as a means of application. There has been a steady change in methods of application, the course spray applied in the hatchery. A modified version of the spray has since developed the "Gel Sprayer" a viscous pressured material is put through a perforated tube. The most recent administration intervention is via the 18-19 day incubated embryos. All application methods for the live vaccines are effective; however, they are only as effective as the birds ingesting the infective oocysts.

EFFICACY OF A LIVE *E. COLI* VACCINE IN COMMERCIAL LAYERS IN MEXICO

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ABSTRACT

Escherichia coli is known as one of the most important bacteria in the poultry industry. It can cause severe losses either as a respiratory (airsacculitis) or reproductive (salpingitis) pathogen. It has been shown there is an increase in antimicrobial resistance for this bacterium; therefore, an alternative control should be approached.

A licensed live *E. coli* vaccine has proven to reduce losses in commercial layers where mortality has been high due to different respiratory factors. Strains of *E. coli* were identified in different complexes. The purpose of this presentation is to show the results using this vaccine under different conditions in the most important production areas of the country.

INTRODUCTION

Escherichia coli infection is often the cause of peritonitis in long lived birds such as commercial layers. Pathogenic *E. coli* are ubiquitous in the environment and can cause airsacculitis, pericarditis, peritonitis, salpingitis, and synovitis in long live birds. This causes decrease in production, which causes significant economic loss to the producer.

Different trials have been carried out in order to prove the efficacy of the *E. coli* modified live vaccine. Four commercial layer flocks were selected to show the results. These flocks are from different parts of the country with different environmental conditions.

MATERIAL AND METHODS

West site. Two hundred and forty thousand (240,000) commercial layers, housed in automated equipment (six levels) with automated feed delivery**,** automated egg-gathering, and automated manure removal**.** Field surveys were carried out in order to isolate and serotype the causative agent.

Birds were vaccinated at 6 and 16 weeks of age via oral route with a commercial 078 modified *E. coli* vaccine. Seven days after vaccination no antibiotic was administered.

North center site. One flock of 59,567 (A) was vaccinated with modified *E. coli*; two flocks, 118,632 (B) and 60,082 (C) were unvaccinated. Control groups B and C had 20.19% and 23.25% mortality while the vaccinated group had 16.05%. Regarding eggs per hen housed, group B had 251, group C 239 and group A 272.

North west site. This company has a recurrent *Mycoplasma* problem. The trial was carried out in two flocks with A) 10,353 and B) 18,621 commercial layers have a final mortality of 11.93% and 15.96%, respectively. The two flocks received a five day treatment of enrofloxacin, and the unvaccinated flock (B) four treatments of lincomycin + streptomycin. Vaccination was done at four and 16 weeks of age.

Southeastern site. A field test was done in two flocks of 23,074 (A) and 24,806 (B). In this site we were able to carry out field surveys in order to identify serotypes; no virulence gene tests were done. Different serotypes were identified from the *E. coli* isolates. From these isolates, 18.75% belong to O 109; 18.75% to O 103; 12.5% to O 18 and O 91 respectively and 6.25% to serotypes O 91, O46, O8, O9, O64, O88, and OR. The main difference in these flocks was mortality – 24.36% vs. 13.02% in the vaccinated flock

RESULTS AND DISCUSSION

The common parameter that we found in vaccinated flocks was the reduction of mortality. These results have an impact as well in eggs per hen housed and feed intake. Another important feature to evaluate is the reduction of antibiotic use. In previous findings of Rosario and associates multi-resistant patterns were observed in *E. coli* strains in Mexico, showing an increase in resistance against common antimicrobials. Control measures for *E. coli* have depended on therapeutic use of antibiotics, and this through years of abuse, has caused a selective pressure for antimicrobial resistance. The use of a live modified *E. coli* vaccine is an alternative way to control this disease.

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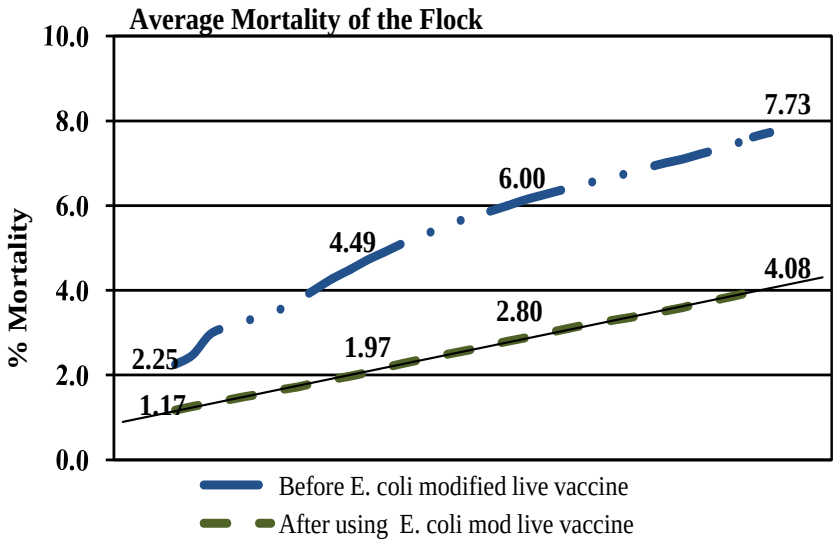
Table 1. Serotypes identified (west site).

Origen	Serotype	Virulence Genes
Yolk Sac	O1	tsh
Lung	O2	negative
Yolk Sac	06	iss
Bone Marrow	O82	negative
Yolk Sac	O120	negative
Yolk Sac	O140	tsh
		iss

Table 2. These results show a mortality reduction of 47%.



PROTECTION CONFERRED TO COMMERCIAL BROILERS VACCINATED WITH THE KILLED RECOMBINANT VACCINE K-NEWH5® OR WITH A COMBINED PROGRAM USING THE LIVE RECOMBINANT VACCINE NEWH5® TO THE CHALLENGE WITH RECENT ISOLATES OF HPAIV-H5N1

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SUMMARY

A trial was conducted in commercial broilers vaccinated with the killed recombinant vaccine K-NewH5® (K-rNDV/LS-AIV/HA/H5) constructed with the insertion of an AIV H5 gene derived from an H5N1 subtype isolated in Indonesia in 2006, but also with a combined program using K-NewH5 and a live recombinant vaccine NewH5 (rNDV/LS-AIV/HA/ H5) constructed with an AIV H5 gene derived from an H5N1 virus isolated in Vietnam in 2004. Results of the challenge with any of the two HPAI viruses isolated in Indonesia in 2007 and in 2009 indicate that commercial birds vaccinated only with the killed recombinant vaccine survived in at least 80% and birds vaccinated with the combined program using the killed and the live recombinant vaccines survived in more than 90%. It can be concluded that the insertion of local AIV genes in the recombinant vaccine is useful for the protection of commercial birds to the challenge with recent isolates of HPAIV-H5N1.

INTRODUCTION

Inactivated whole avian influenza (AI) virus vaccines (K-AIV) have been used since 1978 to control AI viruses in turkeys, breeders, layers, broilers, and fighting cocks. For the Asiatic outbreak in 2004 (H5N1), K-AIV vaccines have been used extensively in all kind of domestic birds, including guinea fowls, partridges, pheasants, quail, and different species of domestic ducks and geese. They are administered individually by the subcutaneous (SQ) or the intramuscular (IM) routes at one week of age and on, and are used with the intention to protect birds against death and clinical disease, but also to stop or diminish field virus replication and excretion. Killed vaccines are preferred for long life birds.

Live and inactivated recombinant Newcastle disease virus-avian influenza H5 vaccines (rNDV-AIV/HAI/H5) were developed to protect commercial birds with the advantage of a DIVA system. This kind of vectored virus allows the production of safety vaccines containing any subtype of AIV HA gene,

without the need of a whole active AIV, eliminating any kind of risks.

Asiatic H5N1 AIV have been changing fast since the original virus from Guangdong in 1996, with 10 main clades, several subclades and emerging variants within the different countries. The need of homologous vaccines has been demonstrated with this Asiatic subtype of AIV.

This is the report of a study done to determine the protection to death of commercial broilers after a single injection of a killed recombinant vaccine or after the use of a combined program using killed and live recombinant vaccines with HA genes derived from Asiatic HPAIV-H5N1, when challenged with recent isolates of HPAIV-H5N1.

MATERIALS AND METHODS

Birds. Newly hatched commercial broilers were obtained directly from the hatchery and housed in isolator houses.

Vaccines. The recombinant virus containing the HA insertion of an Indonesian AIV (H5N1-2006) used for the killed vaccine was prepared to contain a viral titer of $10^{9.3}$ mean chicken embryo infective doses (CEID50)/mL with a minimum 520 HA units (HAU)/mL. Inactivation was performed using formaldehyde to a final concentration of 0.15%. The vaccine was prepared as water-in-oil emulsion.

The recombinant virus containing the HA insertion of a Vietnamese AIV (H5N1-2004) used for the live frozen vaccine was prepared to contain a viral title of $10^{8.1}$ CEID50/mL with a minimum 520 HAU/mL.

Experimental procedure. Birds were vaccinated at 14 d of age. The first group received 0.5 mL of K-NewH5. The second group received 0.5 mL of K-NewH5 and 0.03 mL of NewH5®. A third group remained unvaccinated as control group. At 21 d PV (35 d of age), birds were divided in two groups containing 20 birds each. One group was challenged with a HPAIV (H5N1) strain isolated in 2007 and a second group with a HPAIV (H5N1) strain isolated in 2009. In both cases, the challenge was done using $10^{4.0}$ $CEID_{50}$ by the intramuscular (IM) route. Birds were observed for 14 d PC. At this time, survival birds were humanely euthanized and incinerate.

RESULTS

For the challenge with the AIV isolate from 2007, birds vaccinated only with the killed recombinant vaccine were protected in 80% and birds vaccinated with the combined program were protected in 100%, while in the unvaccinated control group the mortality was 100% within three days PC showing all signs and lesions characteristic of the virus.

For the challenge with the AIV isolate from 2009, birds vaccinated only with the killed recombinant vaccine were protected in 85% and birds vaccinated with the combined program were protected in 90%, while in the unvaccinated control group the mortality was 100% within two days PC showing all signs and lesions characteristic of the virus.

DISCUSSION

Results of this trial indicate that commercial broilers vaccinated with K-NewH5 prepared with an HA gene obtained from Indonesia in 2006 were well protected against the effects of both HPAIV-H5N1 isolated in 2007 and 2009, but that the combined program using K-NewH5 plus NewH5 prepared with an HA gene obtained from Vietnam in 2004 improves the protection to mortality.

PROTECTION CONFERRED TO COMMERCIAL CHICKENS AND DUCKS VACCINATED WITH K-NEWH5® TO THE CHALLENGE WITH A HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS SUBTYPE H5N1

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SUMMARY

A trial was conducted in commercial ducks and commercial chickens vaccinated with the killed recombinant vaccine K-NewH5® (rNDV/LS-AIV/HA/H5) constructed with the insertion of an AIV H5 gene derived from an H5N1 subtype isolated in Vietnam in 2004. Birds were vaccinated once, and at 21 d PV were challenged with a HPAIV-H5N1 clade 2.3.4 isolated in 2008. Results indicate that more than 95% of vaccinated ducks and chickens survived the challenge. It can be concluded that the insertion of local AIV genes in the recombinant vaccine is useful in both ducks and chickens susceptible to the local HPAIV-H5N1 challenge.

INTRODUCTION

Inactivated whole avian influenza (AI) virus vaccines (K-AIV) have been used since 1978 to control AI. They are administered individually by the subcutaneous (SQ) or the intramuscular (IM) routes usually at one week of age and on, and are used with the intention to protect birds against death and clinical disease, but also to stop or diminish field virus replication and excretion. Killed vaccines are preferred for long life birds.

Live and inactivated recombinant Newcastle disease virus-avian influenza H5 vaccines (rNDV-AIV/HA) were developed to protect commercial birds with the advantage of a DIVA system. This kind of vectored virus allows the production of safety vaccines containing any subtype of AIV HA gene, without the need of a whole active AIV, eliminating any kind of risks.

Asiatic H5N1 AIV have been changing fast since the original virus from Guangdong in 1996, with 10 main clades, several subclades, and emerging variants within the different countries. The need of homologous vaccines has been demonstrated with this Asiatic subtype of AIV.

This is the report of a study done to determine the protection from death of commercial broilers and ducks after a single injection of a killed recombinant vaccine prepared with HA genes derived from Asiatic HPAIV-H5N1 when challenged with recent isolates of HPAIV-H5N1.

MATERIALS AND METHODS

Birds. Newly hatched commercial broilers and ducks were obtained directly from the hatchery and housed in isolator houses. Twenty birds were used in each group.

Vaccines. The recombinant virus containing the HA insertion of a Vietnamese AIV (H5N1-2004) used for the killed vaccine was prepared to contain a viral titer of $10^{9.3}$ mean chicken embryo infective doses (CEID50)/mL with a minimum 520 HA units (HAU)/mL. Inactivation was performed using formaldehyde to a final concentration of 0.15%. The vaccine was prepared as water-in-oil emulsion.

Experimental procedure. Birds were vaccinated at 14 d of age with 0.5 mL of K-NewH5. A second group remained unvaccinated as control group. At 21 d PV (35 d of age), birds were challenged with a HPAIV (H5N1) strain isolated in 2008, using $10^{4.0}$ $CEID_{50}$ by the intramuscular (IM) route. Birds were observed for 14 days PC. At this time, survival birds were humanely euthanized and incinerate.

RESULTS

Vaccinated chickens were protected against mortality in 95% and vaccinated ducks were protected in 100%, while in the unvaccinated control groups the mortality was 100% for chickens and 80% for ducks, showing all signs and lesions characteristic of the virus.

DISCUSSION

Results of this trial indicate that commercial broilers and ducks vaccinated with the K-NewH5 prepared with an HA gene obtained from Vietnam in 2004 were well protected against the effects of a HPAIV-H5N1 clade 2.3.4. isolated 2008.

LESSONS FROM HPAI H5N1 IN SOUTH AND SOUTHEAST ASIA

David M. Castellan

INTRODUCTION

Asia contains approximately one-third of the world's human population and three high-risk areas for newly emerging or re-emerging infectious diseases identified by international animal health and human health organizations which include the Amazon Basin, the Congo Basin, the Indo-Gangetic Plain, the Greater Mekong Sub-region, and the Indonesian archipelago. Nipah virus, SARS, and HPAI H5N1 are significant examples of newly emergent diseases discovered in Asia. Eighty-one percent of all officially reported HPAI H5N1 events (10,521 of 12,978) in avian species since January 1, 2004 have occurred in Asia (1).

HPAI H5N1 has had significant social, economic, political, trade and health impact on human and animal health in south and southeast Asia. The World Health Organization currently reports 256 deaths resulting from 375 reported cases in humans from 2003 until August 2010 due to H5N1, a 68.3% case fatality rate in south and southeast Asia (9). The human case fatality rate in Indonesia is 82.5%, one of the highest globally. Exposure is mainly considered to occur through high viral dose due to close contact with poultry; however, some studies indicate possible environmental transmission routes from fresh water sources.

The macro-economic effects of HPAI H5N1 are significant for poultry exporting countries such as Thailand where the disease reduced gross domestic product by only 0.5% to 1.5%, however agricultural growth in that country was reduced by one-half in 2004 (6). Micro-economic and social effect on villages and families has been significant, particularly for smallholder farmers. For example, poultry production in West Bengal, India accounts for 7% to 10% of total household monthly income of $48 USD per month. Total loss per household related to HPAI was calculated at $31.60, not including losses to due periods without poultry (reported range: 90 – 170 days) and without production following depopulation (3).

CHALLENGES

Sixteen different clades or subclades of H5N1 have been identified from 12 countries in south and southeast Asia. Clade 2.3.4 is currently the most common type found in seven of eight countries in east and southeast Asia and 11 clades have been reported. Clade 2.2 predominates in South Asia with the recent inclusion of clade 2.3.2.

Agent pathogenicity is associated with many factors including clade type, species, age, use of vaccination and management. Clade 7 is less pathogenic under field conditions for poultry. Outbreaks in ducks continue in some areas and clinical signs and mortality are commonly detected in young ducks from outbreaks in the region when overt epidemics are not detected and so act as one indicator of hidden infections in poultry populations at large. Vaccination in ducks has been associated with reduced virus shedding and transmission experimentally and empirically under field conditions, particularly in Viet Nam where the number of human deaths have declined.

Selection pressure on the virus is being assessed using antigenic cartography particularly in Indonesia and Viet Nam. China is currently phasing out recombinant Re-1 vaccine in favor of Re-5 vaccine, which offers improved protection against common clades. Blanket vaccination programs are recommended with a more targeted vaccination programs (4) based on operational research currently being pioneered in Viet Nam through the GETS project. Proper collection and isolation of H5N1 from both wild and domesticated ducks remains a challenging aspect of vaccine monitoring.

Understanding of host factors has improved although only a limited number of longitudinal studies of H5N1 transmission in duck flocks have been conducted. Individual ducks are considered to shed for only a brief period and 68.8% of flocks contained shedding ducks when flock mortality was reported in Java, Indonesia (2). Endemic diseases of ducks such as duck plague remain largely under-reported in Asia. Transmission of the virus within poultry flocks varies by production type. Flock management and density influence the basic reproductive rate of infection and secondary cases within back yard, fighting cock, egg layer and broiler flocks (7). The role of wild birds is significant from the perspective of virus introduction but is considered of limited significance in transmission among susceptible avian species. Development of science-based surveillance methods in wild birds and interpretation of results remain difficult. Farming of wild birds for economic profit and satisfying food preferences poses an ongoing risk for poultry in some countries.

The interface between natural and man-made agro-ecological systems including natural areas, cropland, domestic animals and human activity is a key to understanding disease emergence, including H5N1. The role of the environment in virus transmission and

persistence is important, yet largely undiscovered aspect of the epidemiology of HPAI H5N1 subtype. Carcass disposal methods remain challenging during the rainy season and contamination of standing and flowing water sources are potential sources of virus for animals and humans. Technical limitations in study design have hampered field evaluation of the survival of viruses although evidence of H5N1 virus by RT-PCR has been found in and on various environmental surfaces in villages within 12 days of recorded outbreaks (8).

Human factors driving the emergence of H5N1 include immigration, cross-border trade and the close inter-relationships between highly organized commercial poultry units and village/backyard raising systems and poultry marketers play a key role. Free grazing duck systems are being re-structured to reduce human-mediated transmission. Limited infrastructure in epidemiology and laboratory diagnostic capacity does not allow for early detection and rapid response to disease and the majority of countries lack veterinary extension services. Political will and perception of animal owners under various cultural conditions influence the perceptions of "risk" and the incentive to change under conditions where villages and families are stressed economically and socially. Transparency of governments in reporting and sharing scientific data including molecular epidemiology offer both challenge and promise. Farmers have strong economic incentives to capture and/or raise captive wild ducks and other wild animal species to meet consumer demand.

SOLUTIONS

Addressing high-risk practices requires a comprehensive technical, social, cultural and economic approach. Multi-disciplinary and multi-sectoral efforts are being taken to develop plans and strategies for action while promoting a balanced and holistic approach to disease control and protecting livelihoods and sustainability. FAO and its partners are collaborating and coordinating attention on H5N1 and other emerging infectious diseases as follows: Conducting rapid assessments and technical assistance during outbreaks; forecasting and mapping disease hot-spots; building capacity and networks in epidemiology and laboratory diagnosis; conducting operational research to develop more targeted surveillance and vaccination strategies including market chain analysis and cross-border risk mitigation; assisting countries to develop contingency plans; and supporting flexible and country-specific restructuring of poultry production. Community engagement and relevant communication strategies are also being developed to understand and modify human behavior at the community level.

CONCLUSIONS

The need for animal protein in Asia will continue to lead global demand in the coming years. Holistic approaches are used to more effectively deal with HPAI H5N1 and other emerging infectious diseases in Asia. Both technical and human aspects must be addressed including a better understanding of the epidemiology, economic motivation, public-private-partnerships, prevention-minded restructuring of poultry production systems. Both public and private sectors require long-term capacity building and training at individual and institutional levels that include poultry extension and medicine, epidemiology, laboratory diagnosis as well as economics, sociology and communications. Advocacy with governments and industry is a key component of this building process. Epidemiological operational research providing objective indicators is useful to support sound government policies for H5N1 and to support sustainable poultry infrastructure in south and southeast Asia.

ACKNOWLEDGEMENTS

Sincere appreciation is expressed to FAO colleagues at regional and country offices who provided information provided and to national governments for their ongoing collaboration.

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EPIDEMIOLOGY OF AVIAN PATHOGENIC *ESCHERICHIA COLI* (APEC) IN A FINISHING MALE TURKEY FARM: LONGITUDINAL SURVEYS OF THREE CONSECUTIVE PRODUCTION CYCLES

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The purpose of this study was to investigate the epidemiology of APEC (avian pathogenic *Escherichia coli*), in a finishing male turkey commercial farm, conducting longitudinal surveys of three consecutive production cycles. The diversity and the distribution of APEC strains during the production cycles were examined using microbiological and molecular techniques. APEC isolates were serotyped, assessed for the presence of virulence-associated genes (pathogenic potential – pathotype) and for resistance to antibiotics (resistotype). Random amplified polymorphic DNA (RAPD) was applied to analyze their genetic relationship. Strains of high genetic homology were grouped into the same RAPD cluster (RAPD type).

Sampling included, when colisepticemic or articular lesions were observed, viscera and joints from day one until 14 weeks.

In the first cycle, APEC O78 was the most prevalent serotype with all isolates sharing the same resistotype and pathotype. Seven of them were included in the same RAPD cluster indicating high genetic similarity. In this cycle, APEC O111 was detected and this represents, to our knowledge, the first isolation of this serotype in turkeys.

In the second cycle, APEC O2 isolates predominated at the beginning, while O78 strains appeared later until the end of the survey. APEC O2 strains were classified in two resistotypes, same pathotype and RAPD type. APEC O78 belonged to three resistotypes, same pathotype and three RAPD types. APEC O78 strains, differently from serotype O2, were detected from both colisepticemic viscera (such as brain, pericardium, and lungs) and joints. The articular tropism of this serotype is unique as it was observed only in APEC O78 strains of this cycle and of the previous one. Moreover, RAPD molecular analysis identified a specific articular O78 cluster, including two strains, which was different from the clusters of colisepticemic strains. RAPD analysis also demonstrated high genetic similarity between APEC O78 strains of the first and of the second cycle.

The third cycle was characterized by the exclusive isolation of APEC serotype O2 but, unlike the previous cycle, strains weren't detected until week 10. No articular strains were isolated. All strains shared the same resistotype, pathotype and RAPD type. The evaluation of molecular similarity by RAPD, among APEC O2 strains of the second and of the third cycle, revealed two distinct types with low genetic homology, concluding that these strains are not genetically related. They also did not share common resistotype and pathotype.

This study clearly demonstrated the heterogeneous aspect of APEC isolates detected in three consecutive production cycles in the same turkey farm. APEC strains not only differed between cycles but, also, within the same one. They belonged to various resistotypes and all possessed virulence genes responsible for colisepticemic lesions including arthritis. Their variety in RAPD molecular type, pathotype and resistotype indicated that serotyping is not useful for epidemiology studies if it is not in combination with information derived from other

techniques. Only by matching results of all these methods, it can be demonstrated that APEC strains with different serotype, pathotype, resistotype and RAPD type, could coexist, in multiple combinations, in the same productive cycle.

The findings of this study underline the importance of a prompt evaluation of the antibiotic resistance of APEC strains causing mortality during the production cycle. Moreover, it is suggested that the usefulness of futuristic APEC vaccines, should be evaluated regarding the epidemiology of these bacteria in every different flock situation.

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

RETROSPECTIVE STUDY OF NUTRITIONAL ENCEPHALOMALACIA CASES IN CHICKENS DURING 2009-2010

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Vitamin E is an important nutrient for birds. Vitamin E has several related functions.

One of the most important vitamin E roles is its antioxidant activity, which prevents the oxidative degradation of lipids, thus protecting the cell membrane lipids from breaking down (5). Additionally, vitamin E neutralizes free peroxide radicals, formed from lipid oxidation, which damage the cell structural integrity and causes metabolic disorders (5). The vitamin E antioxidant activity protects other fat soluble vitamins, mainly A and D, essential fatty acids, and other highly unsaturated fatty acids from oxidative degradation (2,5). Additionally, vitamin E is necessary for optimal fertility and hatchability, leukocyte and macrophage phagocytic activity, antibody production, and reducing toxicity of some metal such as lead, silver, and arsenic (5). Diets with high levels of unstabilized polyunsaturated fatty acids, which are more prone to oxidation, deplete vitamin E feed content causing vitamin E deficiency (2). Encephalomalacia is a common manifestation of vitamin E deficiency occurring in several species of poultry (6). Other disorders caused by Vitamin E and selenium deficiency are exudative diathesis which occur in chicks, turkey poults, ducklings, and japanese quail chicks; myopathy of the heart and gizzard that happens in chicks and turkeys; and enlarged hocks in turkeys (2). Muscular dystrophy occurs in chickens, turkeys, and ducks with vitamin E and cysteine deficiency (2).

During 2009 and 2010, 15 cases of encephalomalacia related to vitamin E deficiency were diagnosed at the CAHFS, Turlock Branch. Encephalomalacia diagnosis was done by histopathology of brain sections and supported by vitamin E levels in the liver. Age of the chickens ranged from 14 to 27-day-old. Clinical signs included birds reluctant to walk, on their legs or sides, off-balance, paddling, head and leg tremor, opisthotonus, depression, uneven growth, and increased mortality. The most constant gross lesions were hemorrhages in the cerebellum. A few brains were swollen or with pale foci. Most of the birds were moderately dehydrated and with litter contents in their gizzards. Microscopic lesions included areas of encephalomalacia in the white matter of the cerebellum and cerebrum, neuronal necrosis in the granular area of the cerebellum and Purkinje's cells, multifocal hemorrhaging in the cerebellum, and fibrin thrombi. These lesions have been consistently reported in vitamin E deficiency (2,3,4,5,6). Levels of vitamin E in the liver were lower than normal reported for broilers (1).

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SALMONELLA ENTERITIDIS SURVEILLANCE PROGRAMS IN QUÉBEC, CANADA

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In 1997 the Quebec Egg Board created a comprehensive *Salmonella* Enteritidis surveillance program to which all table egg producers must participate. All concerned governmental levels and agencies take part in this integrated surveillance network. A national code of practice for the producers developed by the Canadian Egg Marketing Agency and based on HACCP principles, (called "Start Clean-Stay Clean"), is used as the basis for implementing various control measures.

With a zero tolerance approach and multiple testings at various levels and ages, all positive flocks are rapidly identified. Any breeder pullet flock, breeder flock or commercial pullet flock testing positive to SE is rapidly destroyed because of its spreading potential. In the case of a group D *Salmonella* positive sample from a laying flock, eggs are immediately located and seized at the grading station and a second series of tests is conducted. If results are negative, eggs will be sent back to the market but testing will be conducted monthly thereafter. If results are still positive, the eggs are sent for breaking and pasteurization with a special protocol to handle these SE positive eggs. Hens are prematurely slaughtered to allow for a thorough cleaning and disinfection and empty barn tests. Producers are compensated via a self-administered insurance program.

A traceability system for all shell-eggs has been created and is fully operational allowing for a rapid and efficient recall if necessary. Furthermore, to decrease the risk of a potential outbreak related to shell-eggs from other sources, the program now includes unsettable eggs from Quebec broiler breeder flocks which are purchased by the Egg Board and all sent to breaking.

Since the implementation of these programs, there has been no SE outbreak in the Quebec human population related to the consumption of eggs.

SURVEILLANCE FOR INCLUSION BODY HEPATITIS IN BROILER BREEDERS

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Fowl adenoviruses (FAdVs) are widespread in domestic fowl, and some are associated with inclusion body hepatitis (IBH). FAdVs are transmitted both vertically and horizontally and IBH outbreaks in Canada have resulted in significant economic losses. Previous studies have examined prevalence and exposure to FAdVs associated with IBH in diseased flocks, and characterized viruses involved in outbreaks. However, the prevalence of FAdV infection in clinically healthy flocks is unknown. The objective of the project was to determine the serological status of broiler breeders in Ontario flocks by detecting the presence of antibodies to FAdV serotypes, and to examine shedding of FAdVs in broiler breeder flocks with history of IBH and no history of IBH, by virus isolation and subsequent genotyping.

Seventy-nine Ontario broiler breeder flocks were selected to participate in the serological testing. Fifteen serum samples were collected before the flocks went into egg production. The first submission occurred at 12 to15 weeks and the second submission occurred at 16 to 20 weeks. The samples were screened for the presence of FAdV antibodies by agar gel immunodiffusion, and subsequently by a serotype specific microneutralization test. Flocks negative for FAdV antibodies in the first submission were tested at the second submission. Sixteen flocks were selected from the original 79 to participate in the FAdV shedding study: eight with a history of IBH on the farm and eight with no history of IBH on the farm. Shedding of FAdVs was tested in breeder flocks first before they went into production and then monthly during the lay by virus isolation, avian adenovirus PCR, and genotyping.

Seroconversion to FAdV was positively correlated with flock age, and Ontario broiler breeder flocks before going into production most frequently seroconverted to FAdV08. Isolation of FAdVs occurred in eight of the 16 flocks and included farms with both a history and no history of IBH on the farm. However, isolation of FAdVs from flocks in production was infrequent. Genotyping data will provide a better understanding of the distribution of the various types of FAdVs circulating in Ontario broiler breeder flocks and a comparison of virus isolation and serology results will allow better assessment of the role of horizontal and vertical FAdV transmission than previous studies could. A better understanding of the epidemiology of FAdV infection allows the design of a preventive vaccination program for inclusion body hepatitis.

(The full-length article will be published.)

RUNTING AND STUNTING SYNDROME IN YOUNG BROWN CHICKENS

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Runting and stunting syndrome (RSS), also called malabsorption syndrome (MAS), has been known in broiler chicks since 1970. The onset of this syndrome is typically seen around five d of age, and it is most obvious around two weeks of age. The affected birds are uneven in size and 5% to 15% of the birds can be affected in a flock. Other clinical signs such as diarrhea, lethargy, anorexia, and increased mortality can also be observed. Gross pathology includes watery contents in the small intestine. Microscopically, there is increased cellularity of the lamina propria, blunting and fusion of the villi, and cystic dilatation of crypts. The cause of RSS is not known and it has been attributed to poor nutrition and environment and diseases primarily caused by viruses. Among viruses, reovirus, rotavirus, astrovirus, parvovirus, and other small round viruses have been demonstrated to be associated with RSS. RSS is a common condition described primarily in white broiler chickens but it has not been described in brown chickens.

Several cases of RSS were observed in seven to 24 d old brown chickens submitted to CAHFS that had a history of depression, diarrhea and failure to gain weight and increased mortality. Gross pathology included dehydration, watery contents in the small intestine, and the ceca were occasionally distended with frothy contents. Microscopic lesions ranged from increased cellularity with infiltration of heterophils in the lamina propria and blunting of villi to severe crypt dilatation, which was more prominent in the proximal small intestine. In some cases, viruses including rotavirus, reovirus, and 25 to 30 nm viruses were identified by electron microscopy by a combination of negative stain and transmission electron microscopy (TEM). In one case Rickettsia-like organisms were identified in the cytoplasm of enterocytes by TEM. *Salmonella* spp. was isolated from the intestine in some cases. Attaching effacing *E. coli* (AEEC), coccoid-shaped bacteria attached to the enterocytes, and long segmented filamentous organisms (LSFO) were occasionally observed in the intestine of a few chicks.

CHARACTERIZATION OF A NOVEL CHICKEN ASTROVIRUS ISOLATED FROM INTESTINAL HOMOGENATES OF RSS-AFFECTED CHICKENS

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ABSTRACT

Runting Stunting Syndrome (RSS) has been an important disease in the broiler industry since its first report in 1970s. Isolation of responsible pathogen candidates is fundamental to understand the pathogenesis of the disease and for vaccine development. Although some viruses have been suggested as pathogens, no viruses have been identified as responsible pathogens. Recently, a new chicken astrovirus (CkAstv) was proposed as possible RSS pathogens (6). To isolate a virus which might play a role in RSS filtered intestinal homogenate of RSS-affected chickens was passaged on different cell lines. Using a polyclonal antisera specific for the capsid protein of the new CkAstv specific immune-fluorescence was observed in LMH cells. Exclusion of presence of other viruses that might be related to RSS was conducted by PCR and RT-PCR and serological methods. In addition, the isolate is antigenically different from the known CkAstv 1 and CkAstv 2 as indicated by absence of a specific signal by indirect immunofluorescence IFA and Western blot. The complete genome sequence of new CkAstv was

determined and showed an unusual genomic organization for *Astroviridae*. ORF1a and ORF1b are encoded by two separate open reading frames without an indication of a frame shift site. Comparisons of the amino acid sequences of the three encoded proteins clearly indicate a novel CkAstv from RSS affected chicken.

INTRODUCTION

Runting and stunting syndrome (RSS) has been recognized since the late 1970s. All associations to this disease manifestation have been based on clinical signs. Although management issues play an important role in the manifestation of RSS, infectious agents, namely viruses have been implicated as etiologic agents. Specifically, reoviruses, rotaviruses, parvoviruses, enterovirus-like viruses, adenoviruses, caliciviruses, and a togavirus-like viruses have been observed in the intestines and contents of clinically affected birds by virus isolation and/or electron microscopy (5,3). Avian reoviruses (ARV) are commonly isolated from healthy chickens but are also associated with several diseases, namely viral arthritis and RSS. The relationship of reoviruses in cases of RSS is not well understood. Reoviruses isolated from cases of RSS have been shown to cause lesions in the duodenum, jejunum and ileum with (1,2) or without an associated weight suppression (7). Avian nephritis virus, a recently classified member of the family *Astroviridae* has also been implicated in RSS. Early investigations into the etiologies of RSS identified entero-like viruses in young chicks. A crude inoculum containing these viruses and a reovirus consistently produced slow feathering, fecal changes and depressed weight gains in broilers inoculated at one day of age (3). Experimental attempts to reproduce this disease with these agents have been inconclusive likely due to the fact that the causative agents can not be cultivated. Thus, the etiology of this disease remains undetermined. Viruses implicated in enteric diseases have also been detected by molecular methods in clinically healthy chickens and therefore the clinical importance of these viruses remains unclear (4). No infectious agent has been described so far and only clinical signs, gross lesions and histopathology (cystic enteropathy) can suggest a diagnosis.

MATERIALS AND METHODS

Cells and chickens. A chicken hepatocellular carcinoma cell line (LMH) were cultivated in Dulbecco's Modified Eagles's Medium with 4.5g/L glucose (DMEM-4.5, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Mediatech, Manassas, VA, USA) in the presence of penicillin (100 IU/mL) and streptomycin (100 ug/mL). Commercial one-day-old broiler chickens were obtained from a local hatchery. The breeder flock has not been vaccinated with an autogenous RSS vaccine.

Virus isolation. Gut content from one hundred RSS affected chickens was diluted 1:1 with phosphate buffered solution and homogenized using a blender. The homogenate was aliquoted and stored at -80°C until use. The thawed RSS samples were centrifuged and the supernatant was filtered with a 220 nm filter. The filtered material was incubated with a chicken reovirus-specific antiserum (Charles River Spafas, Franklin, CT, USA), incubated for 1 h at 37°C, and inoculated into sub-confluent LMH cells. Five days after inoculation the cells were freeze/thawed and used for a subsequent passage on LMH cells.

Infection experiments. One-day-old commercial broiler chickens were orally infected with 1 mL of the isolated astrovirus. Appropriate controls were not infected. Five, eight, and 12 d after infection birds were euthanized, weighed, and the duodenal loop was taken for microscopical evaluation. In addition, the gut samples from d 5 post infection were investigated by in situ hybridization for the presence of viral RNA.

Cloning and sequencing. The viral RNA from passage 5 of the virus was purified with the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). The purified RNA was used for the cloning of the full length sequence applying standard procedures. The 5'end of the viral genome was obtained by using the 5'RACE kit (Invitrogen, Carlsbad, CA, USA).

RESULTS

Virus isolation. During passage in LMH cells a virus was detected using a rabbit Astrovirus capsid-specific antiserum (rAstCap, 6) by indirect immunofluorescence. In the infected cell culture cytopathogenic effects were observed typical for avian reovirus. The presence of such viruses was confirmed by indirect immunofluorescence using the chicken reovirus-specific serum. The chicken reovirus was eliminated from the infectious material by end-point titration of the virus supernatant and the absence of reovirus was confirmed by subsequent passages of the obtained supernatants and tested by indirect immunofluorescence. The obtained astrovirus was propagated until passage 5 and used for subsequent experiments.

Growth kinetics. LMH cells were infected with a multiplicity of infection of 1, incubated for 1 h at 37°C and the supernatant was removed. The cells were washed once with serum free medium and overlaid with 1% FSB containing DMEM. Supernatants were immediately taken and at d 1, 2, 3, 4, and 5 p.i. $TCID_{50}$

were determined in LMH cells following standard procedures using the rAstCap serum in an indirect immunofluorescence since the virus did not cause a significant CPE in cell culture. An increase in virus titers were detected as early as 48 h p.i.. The plateau in virus titer was reached 5 d after infection with a $TCID_{50}$ titer of $10^{6.5}$/100 uL. In addition, viral titers were separately analyzed for the supernatant and the cells at d 1, 2, 3, and 4 p.i. Interestingly, the highest virus titers were present in the infected cells at every time point analyzed.

Chicken experiment. Chickens were orally infected with 10^7 infectious virus particles as determined by $TCID_{50}$. Infected chickens showed a reduced weight (up to 20%) in comparison to the control at d 5, 8, and 12 after infection. More interestingly, all chickens infected with the newly isolated chicken astrovirus (CkAStV) showed a signal after *in situ* hybridization using CkAstV antisense viral cRNA-probe in the crypt of Lieberkühn at d 5 p.i. indicating the replication of this particular virus in this region of the gut. In addition cystic enteropathy, the hallmark lesion for RSS, was observed in a number of infected chickens.

Analysis of the viral genome. The full length sequence of the viral genome of the isolated CkAstV was determined by a combination of several molecular techniques. The genome length is 7499 nt excluding the viral poly-A tail. The virus encoded for three open reading frames (ORF1a, ORF1b, ORF2). In contrast to other astrovirus the ORF1b, which encodes for the putative RNA dependent RNA polymerase, contains its own start codon indicating a different replication strategy of the isolated virus. The comparison of the amino acid sequence revealed that this CkAstV showed the highest similarity with 69%, 71%, and 41% to proteins of a duck astrovirus encoded by ORF1a, ORF1b, and ORF2, respectively. The obtained data clearly demonstrate that the isolated virus present a new member of the Family *Astroviridae*, Genus *Avastrovirus*.

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"SPOTTY LIVER DISEASE" – AN EMERGING DISEASE IN FREE-RANGE EGG LAYERS IN AUSTRALIA

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SUMMARY

"Spotty Liver Disease" (SLD) has become a major cause of mortality in egg layers housed predominantly on the ground in Australia. The increased prevalence in recent years appears to be associated with the concurrent increase in free-range and "barn-laid" egg production. Usually SLD has occurred in early lay, causing up to 10% mortality and up to 10% egg production decline for two to eight

weeks. A bacterium is thought to be the causative organism on circumstantial evidence. Antibiotic treatment has usually been successful, but economic loss due to excess mortality and decreased egg production is nevertheless significant. There are only two antibiotics permitted for treatment of egg layers during lay in Australia and one is already becoming less effective against SLD. Various medication and husbandry programs have been only partially successful in preventing SLD. The disease resembles what was described in scientific literature many years ago as vibrionic hepatitis, but whether SLD is the same disease has not been confirmed. A case report of SLD on a multi-age, free-range, egg layer farm is described. The Australian Egg Corporation Limited has recently supported a research project in Australia to try to elucidate the cause of SLD.

INTRODUCTION

Spotty Liver Disease (SLD), "summer hepatitis", and miliary hepatitis are terms used in Australia for a disease of egg laying domestic fowls dying acutely in early lay with multiple, small, cream-colored spots in the liver. Sporadic diagnoses of SLD were made as early as 1996 in "barn-laid" and free-range egg layers (R. Reece, personal communication). While the disease has occurred in meat breeders on litter in sheds, in six to eight week old broilers (R. Reece, personal communication) and very occasionally in caged egg layers, SLD has become more prevalent as the percentage of egg layers housed on the ground has increased in Australia from insignificant levels to approximately 25% of total egg production, which includes 20% free-range and 5% barn-laid production. SLD is now one of the most common "infectious" diseases of free-range and barn-laid egg layers in Australia. The economic importance of the disease is due to increased mortality, egg production drops, and sometimes pale yolked eggs, which are not suitable for sale as free-range whole eggs.

DISEASE DESCRIPTION

The first indication of SLD in a flock is usually increased mortality in early lay, often between 22 and 28 weeks of age. Mortality rates up to 10% for two to six weeks can occur. Daily egg production decreases almost simultaneously up to 10% for two to eight weeks. The disease does not occur in flocks in rearing and has not been reported in male breeders in affected meat breeder flocks. Hens that have recently died are very hot to feel suggesting that they had a fever. Dead birds are in good body condition and in full lay, often with full crops. Soiling below the vent with faeces and urates occurs in some affected birds.

At autopsy, the most noticeable lesion is multiple, small, cream-colored spots in the liver, which is paler than normal and sometimes slightly swollen. Some affected birds appear to be jaundiced with yellow discoloration of the comb, wattles and body fat. A mild enteritis with pink, salmon-colored exudate on the mucosa, a slightly enlarged mottled spleen and excess fluid in the peritoneal cavity and lung are found in some birds.

Microscopic examination of the liver reveals a necrotizing hepatitis, occasionally with heterophil and macrophage infiltration. There is no particular pattern of lesion distribution and usually no hemorrhage as the sinusoidal framework remains intact. Local fibrinoid accumulation occurs and occasionally a laminated thrombus results. Special staining has not detected bacteria or other microorganisms, roundworm larvae or histomonads in the lesions. The histological lesions in the liver are dissimilar to adenoviral hepatitis, Big Liver Spleen disease (avian hepatitis E virus infection), bacterial abscesses, and infections with *Escherichia coli*, *Salmonella*, *Pasteurella multocida*, *Erysipelothrix rhusiopathiae*, *Chlamydophila psittaci*, and *Clostridium perfringens*. *Campylobacter*-like bacteria have been observed in a few affected livers. Although *Campylobacter* spp. have been isolated from normal livers of broilers, the isolation rate from livers of broilers with hepatitis is greater than that from normal livers (1,2). The microscopic lesions do not resemble those due to aflaxtoxin toxicity. Electron microscopic examination of numerous affected livers has not revealed any putative pathogen. In a small proportion of affected birds fibrinoid necrosis around the ellipsoids of the spleen is observed and also non-suppurative peritonitis. Mild enteritis is found in some birds, but the lesions were not typical of necrotic enteritis due to *Clostridium perfringens*.

Culture of livers for aerobic bacteria in a number of laboratories has eliminated such pathogens as *E. coli*, *Salmonella*, *E. rhusiopathiae*, *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and *Pasteurella* spp. as the cause. *Campylobacter jejuni* was isolated by one laboratory from the bile of one affected bird; *Campylobacter coli* has been isolated from livers of affected birds by another laboratory, and *Clostridium sordelli* was isolated from the liver in another case by a different laboratory. Other laboratories have failed to isolate or identify *Clostridium* spp. from affected livers. *Clostridium* spp. has been isolated from the intestinal tract of affected and non-affected flocks.

ETIOLOGY

Circumstantial evidence indicates that a bacterium is the cause of SLD. Treatment with selected

antibiotics results in a rapid response, with the mortality rate decreasing and egg production increasing. Bacteria most commonly proposed as the cause include *Campylobacter jejuni*, *Campylobacter coli*, and *Clostridium perfringens*. Chlortetracycline (CTC) and Linco-Spectin, which contains lincomycin and spectinomycin, are the two main antibiotics used in egg layers to treat SLD, as these are the only two antibiotics registered with a Nil Withholding Period for table eggs in Australia. Amoxycillin has been an effective treatment of SLD in meat breeders. Mortality can cease as early as two days following initiation of water medication. However, the disease can recur shortly after cessation of antibiotic treatment resulting in a repeat antibiotic medication in drinking water. Short-term, in-feed CTC has also been used effectively for treatment in some outbreaks for this reason.

EPIDEMIOLOGY AND PATHOGENESIS

SLD typically occurs in the hotter months of the year. Moist litter is considered to be a precipitator. The possibility that round worm or coccidial damage to the intestinal lining is a predisposing cause has been proposed. Stress factors such as hot-humid weather, overcrowding in shelter houses on the range, inadequate feed space and cannibalism have also been proposed as contributors. The occurrence in only hens in lay and not in males may suggest some hormonal influences. The ingestion of infected faeces may be required, as SLD occurs almost exclusively in hens that are on the ground, either on litter or on range. There does not appear to be any bird strain difference in susceptibility. SLD rarely recurs after the initial outbreak, maybe indicating that immunity can develop. A hypothesis for the pathogenesis is that the causative organism multiplies in the intestinal tract which results in a bacterial toxin causing liver lesions.

TREATMENT AND PREVENTION

SLD responds rapidly to antibacterial treatment, particularly CTC, Linco-Spectin, and amoxycillin. However, apparent resistance has developed to CTC in some flocks, resulting in less successful treatment response despite higher levels of CTC and longer treatment duration times being used.

In-feed additives that have been used with little success to prevent SLD include flavophospholipol, zinc bacitracin, organic acids, probiotics, and a compound containing essential oils.

Enhanced biosecurity and hygiene procedures, including sealing dirt floors of sheds and disinfection of footwear into sheds, are reported to have been successful in preventing SLD in fully-shedded meat breeder flocks.

CASE REPORT

SLD has occurred on a large, multi-age, egg laying farm in the last seven years. The severity of the disease has varied between flocks for no apparent reasons. The initial outbreak was found in 25-week old caged layers, but subsequently occurred within the next 12 months in a barn-laid shed and in several free-range sheds on the multi-age farm. Initially outbreaks occurred in the hotter months of the year, but in recent years SLD has occurred in free-range flocks in early lay throughout the year. There were no further outbreaks in caged layers on the site. Histological examination of livers with multiple, small, cream-colored foci revealed lesions consistent with SLD. Aerobic bacteria have not been isolated from the liver of any affected hens.

One year after the initial outbreak occurred on the multi-age farm, SLD occurred in a small group of young egg layers on a single-age farm contracted by the same egg layer company. This group had colony nest boxes containing sawdust, while all other groups on the farm had traditional single-hole metal nest boxes. This has been the only SLD occurrence on the contract farm, despite the fact that all replacement pullets are reared on litter on the affected multi-age farm and transferred to the contract farm at 15 weeks of age. Nor have there been SLD outbreaks on another multi-age, free-range farm owned by the same company which has slatted floors and automatic collection nest boxes in sheds. All free-range sheds on the affected multi-age farm contained colony nests with sawdust. Recently colony nests were replaced by a roll-away nest system in some sheds on the multi-age farm, but SLD has continued to occur in these sheds.

Many of the outbreaks occurred following sawdust litter on the floor of sheds becoming wet due to rain entry or leaking drinker nipples. In some cases it was found that SLD mortality occurred first in the areas of the shed where the litter was wet. Dusting of lime ($CaCO_3$) under drinker lines and into nest boxes, spraying of the litter with an iodophor sanitizer and weekly dosing of drinking water from a deep bore with iodophor may have delayed the onset and decreased the severity of SLD. There is some indication that increasing the body weight of pullets in late rearing and early lay may be partially protective.

Other measures progressively implemented include installation of fans in sheds, discontinuing beak trimming at 12 weeks of age, better management of coccidiosis immunization, more regular worming with levamisole (every six weeks during lay), improved rodent and fly control, and disinfecting of boots into sheds by dipping in iodophor solution. In-feed flavophospholipol, zinc bacitracin, and essential oils have been unsuccessful in preventing SLD, as has

addition of organic acids to drinking water from a deep bore to reduce the pH.

For the last two years Linco-Spectin has been used for treatment instead of CTC which became ineffective. Because Linco-Spectin is the only other antibiotic that can be used in lay without discarding eggs, there is some urgency to solve the problem of SLD on the affected multi-age farm.

DISCUSSION

Because SLD usually responds rapidly to antibiotic therapy, a bacterium is likely to be involved in the etiology. However, the identification of the bacterium has not been established. Laboratory culture of livers for aerobic bacteria is always advisable if SLD is suspected, as SLD has sometimes been mistaken for fowl cholera and erysipelas.

SLD resembles vibrionic hepatitis, which was first described in the USA in 1954 (3) with the causative agent being proposed as a "vibrio" in 1958 (4). A similar disease occurred in the UK in the late 1980s (R. Reece, personal communication), but was not again reported in the UK until 2003 (5,6) and 2005 (7), perhaps due to increasing numbers of free-range egg layer flocks around this time. The vibrio involved was later renamed *Campylobacter jejuni*. *Campylobacter coli* was the predominant bacterium isolated from the liver and gall bladder of ostriches with a severe necrotic hepatitis (8), although the histopathology is not identical to that found in SLD. Cholangiohepatitis in broiler chickens due to *Clostridium perfringens* (9) has histopathological lesions that differ from SLD. *Clostridium sordelli* caused hepatitis in an ostrich (10). *Heliobacter pullorum* has also been suspected to cause liver lesions in chickens (11).

SLD has not occurred in the last six years in replacement pullets that were reared in close proximity to laying flocks affected with SLD on a multi-age farm and transferred to a contract free-range laying farm, suggesting that some specific predisposing factors are involved in the pathogenesis of SLD and that these factors exist on the affected multi-age farm.

While a clostridial infection/toxin could be the cause of SLD, the histological lesions are not typical, clostridia have rarely been detected in lesions microscopically and in-feed zinc bacitracin has not prevented the occurrence of SLD. The failure to consistently isolate *Campylobacter* from the liver or to detect *Campylobacter* in lesions histologically also suggests that *Campylobacter* may not be the cause.

To date there has been limited research on this disease in Australia which has not determined the etiology or definitive control measures. The Australian Egg Corporation Limited has recently funded a research project at the University of Melbourne to apply culture-free metagenomic studies using high-throughput DNA sequencing to define the etiology.

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A CASE OF H5 LOW PATHOGENICITY AVIAN INFLUENZA IN A LIVE BIRD MARKET IN SOUTHERN CALIFORNIA

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INTRODUCTION

In the event of H5 or H7 low pathogenicity avian influenza (LPAI) detection in domestic poultry California utilizes the Incident Command System and implements the "Initial State Response and Containment Plan for Low Pathogenicity H5/H7 Avian Influenza in Domestic Poultry". Low pathogenic strains of H5 and H7 avian influenzas are of particular concern in the live bird marketing (LBM) system because of the perceived risk of mutation to high pathogenic avian influenza (HPAI) strains of the virus when poultry populations from assorted locations and species are housed together for extended periods (1). Additional concerns are trade sanctions following detection of LPAI and HPAI viruses, spread to the commercial industry and the ability of some HPAI viruses to infect humans (1). In 2004, the live bird market industry in southern California collaborated with the University of California Cooperative Extension and the California Department of Food and Agriculture (CDFA) to create "The Avian Influenza Control Plan" to provide standards for monitoring and detection of avian influenza in custom slaughter live bird markets. Surveillance and inspections of markets and producers is conducted by USDA/CDFA personnel. The plan is designed as a voluntary program for the control of non-H5, non-H7 LPAI. There are currently 32 markets in southern California participating in the program. There are 15 suppliers of various species supplying southern California markets participating in the program.

CASE REPORT

On November 3rd, 2008 a LBM in southern California tested positive for H5 avian influenza. Chickens were present from two suppliers (white broilers and brown pullets). Five chickens from each source were swabbed and pooled in separate vials. Both types of chickens were positive. All southern California markets were negative in October and no other market tested positive in November. All markets were depopulated and inspected on November 4th. The infected market repopulated and the positive result of the November 3rd test was received on November 5th. The market was retested and a H5 AI was detected in chukars by PCR. The market was quarantined immediately. The incident command system was established and the epidemiologic investigation was initiated. The market was depopulated, indemnified and cleaned and disinfected under CDFA/USDA supervision the following week. The method of depopulation was stunning followed by exsanguination supervised by a veterinarian. Environmental testing was conducted. The results were PCR positive and virus isolation negative. It was cleaned and disinfected two more times while waiting for the virus isolation results. Environmental swabs were taken and remained PCR+. When the initial results were returned as virus isolation negative the market was released from quarantine and allowed to sell chickens and turkeys only. The poultry was tested weekly for a month and remained negative and the store was allowed to resume normal operations. Markets sharing the same suppliers as the positive market tested negative in October and November. All markets were negative in December. No indication of illness was evidenced by the infected poultry. All birds appeared bright and alert. Records of deliveries for the previous 90 days were reviewed. Each ranch supplying the market was tested by Foreign Animal Disease Diagnosticians (FADDs). All ranches had a minimum of 30 birds swabbed and bled. The spent hen supplier provided records of his route and the sources of the spent hens and the source ranches were tested. The chukar and duck suppliers were tested multiple times. Surveillance was expanded to ranches associated with suppliers but that had no direct contact with infected markets. There were no positive results. The initial H5 PCRs in the chickens and the chukars were confirmed by National Veterinary Services Laboratory (NVSL) but virus was not isolated. No source was identified but a LBM has many inputs. This is a very busy store with close to 20 culturally diverse employees. A multitude of customers, inspectors from various government agencies, rendering pick- ups, and feed deliveries as well as many different suppliers supplying birds on a daily basis in approximately a 4,000 square feet area of store, parking lot and delivery bay creates a situation where disease can be introduced from many sources.

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TELLING THE REAL TRUTH ABOUT BIOSECURITY

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Compliance with biosecurity measures is poor in all types of animal productions around the world. It is essential to define strategies to improve the implementation of biosecurity measures. Different approaches have been studied in human medicine. They include daily observations and feedback to employees, training programs, and the presence of an observer. These strategies have been shown to work, but only for a short period of time. This study evaluated the value of audits and cameras on compliance with biosecurity measures required when entering and exiting poultry barns on 24 poultry farms in Quebec, Canada. The effect was determined in the short term (first two weeks) and in the medium term (six months later). Compliance was evaluated using hidden cameras.

Production types were meat-type farms (chickens or turkeys), commercial layers and breeder farms. The inclusion criteria were: Having at least one employee and a biosecurity protocol in place. The idea was to have farms where we can expect some traffic. One barn was randomly chosen for each farm. Each grower was met to sign confidentiality agreements and to confirm the farm's biosecurity protocol currently in place in order to produce and install a laminated poster listing entry and exit biosecurity measures at the entrance of the selected barn. All the farms were randomly assigned to three groups (control, audit and camera) of eight farms. However, the distribution was done to make sure that each production type was present in each group. For the control group, there was no intervention, other than installing the laminated poster with the grower's list of required measures. A hidden camera was installed in the entrance of the barn. For the audit group, there was also a hidden camera in the entrance, 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 audits over a six month period and they consisted in evaluating the availability and feasibility of biosecurity measures. Employees also got to assess their own compliance, and biosecurity principles were discussed with them during each visit. Finally, 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. A hidden camera was also present. All the farms were followed for two weeks after setting up the surveillance system (short term viewing) and six months later, for another two weeks corresponding to the medium term viewing.

For the short term assessment, there were 1517 visits done by 176 different individuals (68 full-time employees, 26 part-time employees, 79 visitors, and three farm co-owners). There were 145 men and 31 women. For the medium term assessment, there were 1231 visits done by 169 different individuals (74 full-time employees, 30 part-time employees, 60 visitors, and five farm co-owners). Results for the short term assessment are presented for each biosecurity measure. Statistical analyses were performed using MLWiN software (MLwiN 2.22) with RIGLS method, logit link function, first order penalized quasilikelihood estimation (second order when possible) and a binomial distribution. Multilevel logistic regression models with random effects for individuals (level two) and farms (level three) were performed using a backward stepwise method. The final models with random and fixed effects are presented with p-values, coefficients, standard errors, odds ratios, confidence intervals and interpretations. For this presentation, only short term results are presented and discussed.

1) **Log book.** There were 106 visits done by 75 different individuals on 20 farms that required signing a log book. The only variable that was significant in the final model was the presence of an observer. The coefficient and the standard error were -1.518 and

0.583, and the odds ratio was 0.22 (0.07-0.69), meaning that there were almost five times fewer chances to sign the log book for visits with an observer (*P*=0.01).

2) **Areas at entrance (outside or contaminated area; inside or clean area).** There were 1070 visits done by 132 different individuals on 23 farms that required respecting areas at entrance. Visits without boots were excluded from this analysis because areas could not be respected. The only variable that was significant in the final model was the type of area delimitations. The coefficient and the standard error were 2.203 and 0.959, respectively and the odds ratio was 9.1 (1.4-59.3), meaning that there were nine times more chances of respecting the clean and the contaminated areas at entrance if the delimitation was a physical barrier (e.g., bench) compared with a red line (*P*=0.02).

3) **Areas during the visit.** There were 632 visits done by 82 different individuals on 18 farms that required respecting areas after the entrance and before the exit. Visits without boots were excluded from this analysis because areas could not be respected. The only variable that was significant in the final model was the presence of an observer. The coefficient and the standard error were -0.696 and 0.343, respectively and the odds ratio was 0.50 (0.26-0.98), meaning that there were two times fewer chances of respecting the clean and the contaminated areas during the visit for visits with an observer (*P*=0.04).

4) **Areas at exit.** There were 1071 visits done by 132 different individuals on 23 farms that required respecting areas at exit. Visits without boots were excluded from this analysis because areas could not be respected. The only variable that was significant in the final model was the type of area delimitations. The coefficient and the standard error were 1.560 and 0.694, respectively and the odds ratio was 4.8 (1.2-18.5), meaning that there were almost five times more chances of respecting the clean and the contaminated areas at exit if the delimitation was a physical barrier compared with a red line (*P*=0.03).

5) **Coveralls.** There were 681 visits done by 91 different individuals on 16 farms that required coveralls. The variables that were significant in the final model were the moment of the visit and the presence of the grower. For the moment of the visit, the coefficient and the standard error were 2.051 and 0.307, respectively and the odds ratio was 7.8 (4.3-14.2), meaning that there were eight times more chances to put on coveralls for visits in the morning compared to the afternoon (*P*<0.0001). For presence of the grower, the coefficient and the standard error were -1.401 and 0.486, respectively and the odds ratio was 0.25 (0.10-0.64), meaning that there were four times fewer chances to put on coveralls for visits when the grower was present (*P*=0.004).

General comments. For the short term period, audits did not have any impact on compliance and visible cameras only had an impact on changing boots (OR=9.6; 1.9-48.4). Other variables were associated with compliance to some biosecurity measures, such as duration and time of the visit, presence of the grower or an observer (behavior of observers seem to have a significant impact), design of the entrance and number of barns. We have to provide training material to make sure that everyone understands biosecurity principles. It is also essential to design entrances that facilitate daily compliance.

Table 1. Modelization results for the variable "using boots according to requested protocol."

Variables	P-value	Coefficients and standard errors	Odds ratios and confidence intervals	Interpretations
Study groups 1=control 2=direct audit[1] 3=camera 4=indirect audit[1]	0.03	**2 vs. 1** = 0.606 (0.817)	1.8 (0.37-9.1)	There were almost 10 times more chances to put on boots when there was a visible camera in the entrance of the barn compared to the control group.
		3 vs. 1 = 2.262 (0.825)	**9.6 (1.9-48.4)**	
		3 vs. 2 = 1.656 (0.901)	5.2 (0.90-30.6)	
		4 vs. 1 = -0.100 (0.852)	0.90 (0.17-4.8)	
		4 vs. 2 = -0.706 (0.675)	0.49 (0.13-1.9)	
Barns	0.01	1.697 (0.675)	**5.5 (1.5-20.5)**	There were five times more chances to put on boots when there were more than five barns on the site.
Observer	0.002	-0.770 (0.250)	**0.46 (0.28-0.76)**	There were two times fewer chances to put on boots for visits with an observer.
Duration of visit[2] 1=<5 min 2=5-17 min 3=17-54 min 4=≥ 54 min	<0.0001	4 vs. 1 = 3.581 (0.337)	**35.9 (18.6-69.5)**	There were 12 to 36 times more chances to put on boots for visits lasting longer than five min. There were three times more chances to put on boots for visits lasting longer than 17 min.
		3 vs. 1 = 3.480 (0.299)	**32.5 (18.1-58.3)**	
		2 vs. 1 = 2.490 (0.282)	**12.1 (6.9-21.0)**	
		4 vs. 2 = 1.091 (0.326)	**3.0 (1.6-5.6)**	
		3 vs. 2 = 0.990 (0.298)	**2.7 (1.5-4.8)**	
		4 vs. 3 = 0.101 (0.313)	1.1 (0.60-2.0)	

[1]Individuals who visited farms from the audit group were classified in two groups: Direct audit when they participated to all audits and indirect audit when they were not present when audits were performed.

[2]The duration of all visits were divided in four equal groups (quartiles) and categories were built accordingly.

DISEASE SPREAD, WE KNOW A LOT… BUT NOT THAT MUCH

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Emerging and re-emerging animal diseases over the last 10 years have raised much awareness as to the importance of biosecurity. Several countries have or are developing national standards for biosecurity. It is recognized that heterogeneity in both geographic location and transmission mode may affect our ability to predict the potential effectiveness of control measures. Our ability to predict and/or protect is dependent on the true efficacy of biosecurity measures. To study the impact of biosecurity practices and the consequences of the uncertainty in biosecurity parameter estimates on disease spread, a computer simulation model of poultry production was used. The model is described in a companion poster. To provide estimates of parameter values for the model, a literature review was undertaken. We then present preliminary results for avian influenza.

MATERIAL AND METHODS

Literature review. Our review was limited to diseases considered of importance by the poultry industry in Quebec, Canada. Thus information on the transmission of the following diseases was researched: infectious bursal disease (IBD), infectious bronchitis virus (IBV), inclusion body hepatitis (IBH), Newcastle disease virus (NDV), avian influenza (AI). Given space limitation, we limit this paper to more interesting aspects of IBD, IBV, IBH, and AI, providing references for specific points.

Simulation model. At the time of writing, model development was not finished. For illustrative purposes we present some observations made on an initial beta model programmed in the NetLogo simulation environment developed as a proof of concept. The disease considered is low path avian Influenza spreading silently. Within flock dynamics is modeled after Savil *et al*. (7). We included 300 flocks (one flock per farm) dispersed at random over a 70x40 km area. We considered different mixtures of bird types, two hatchery representatives, four feed-mill representatives, and vehicles for feed delivery to these farms. The probability of a visitor becoming contaminated or contaminating a site as the result of a visit was set at 5% in the absence of biosecurity measures. The probability of a delivery truck becoming contaminated or contaminating a site was set at 2%. Representatives and trucks remained contaminated only for 24 hours. Biosecurity was randomly allocated to values between 1 and 5 for each flock. At every visit a random number was generated and the proper comparisons made to determine whether or not contamination occurred.

RESULTS

Literature review. For IBD the only significant work on transmission is that of Benton *et al*. (1). Flensberg *et al*. (3) associated certain hatcheries, age of parent birds and a certain feed mill with the introduction of acute clinical infectious bursal disease among Danish broiler chickens in 1998. Sanchez *et al.* (6) found cases were more likely to occur during a short period of time and over relatively short distances, indicating that local factors facilitated the spread of the virus.

The case of IBV is even more interesting in that our knowledge of transmission is actually poor. Hofstad (4) writes that the frequency of airborne spread is unknown. Cavanagh in his 2007 review (2) states "The virus is highly infectious, *presumed* (our emphasis) to spread by aerosol as well as by mechanical means." On the other hand IBV is one of the very few poultry diseases for which an estimate exists for the within flock transmission rate (8).

Vertical transmission of IBH is recognized as important and several publications do support this claim. On the other hand, horizontal spread of the virus is also deemed important because the virus is present in feces, in the tracheal and nasal mucosa and in kidneys. Therefore, the virus could be transmitted in many excretions. It is also stated that aerial spread between farms would not be important, except when dust is created when cleaning poultry houses.

Avian influenza, especially because of its importance from a human perspective, has generated over the recent years a great wealth of information. Several models have been published as described in the companion paper. Most models have put emphasis on direct and indirect contacts, aerosol not being considered important. Several models for within flock transmission have been also published with parameter estimates.

Simulation model. For a given area, the proportion of types of production affects the

probability of an epidemic to occur. If only broiler chickens are considered, the frequency of an epidemic is of the order of 8%. When turkey and layers flocks are added, the frequency increases to 60%. This behavior is in agreement with the fact that AI is more often observed in layers and turkeys. These flock types being of longer duration allow for the disease to become well established and also offer more opportunities over time for effective contacts leading to the transmission of the disease.

Once a simulated epidemic is started several types of patterns may develop as shown in figure 1: Gradual increase to low prevalence of 20% or high prevalence at 40%; a two phase outbreak; an acute outbreak in the case of aerosol transmission. On examining the output data of the simulations, we could see that the epidemic was triggered mainly by one of the following: infection of a turkey or layer flock, a switch to the clients of another hatchery, and/or feed company having a boosting effect on the epidemic. Adding aerosol transmission caused the epidemic to quickly rise to a prevalence of 60%.

CONCLUSION

Overall, the published literature provides ample documentation as to the means of spread of diseases. Unfortunately, we lack measures for several key parameters, for example the probability of a visitor contaminating a flock given a previous visit in a contaminated flock. Thus, most models rely on "expert opinions" which simply reflect the current beliefs of these experts. Therefore, policies and current risk analyses derived from simulation models are essentially based on our currents beliefs and not on data. On the other hand, for a very few diseases, we do have some estimates of within flock transmission parameters. In other words, we may know a lot, but really not that much.

Nevertheless, the beta model demonstrated that even with relatively simple assumptions, patterns of epidemic may be quite diverse and the patterns produced do have similarities with patterns observed in the real world.

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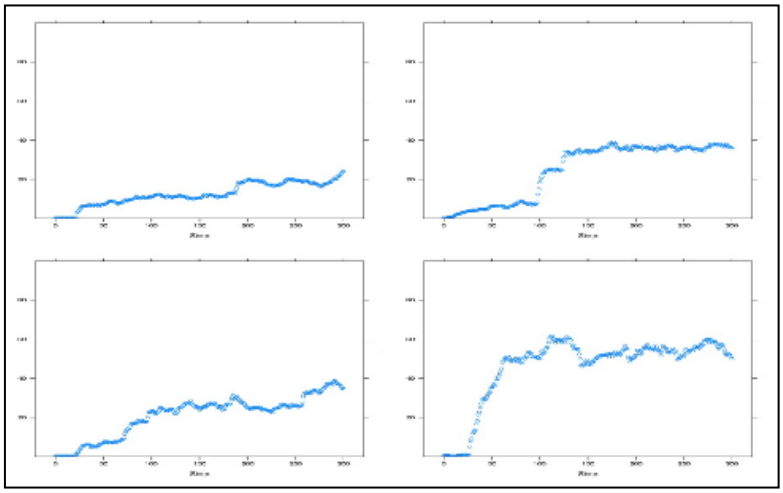
Figure 1.



BIOSECURITY CHALLENGES ON A MULT-SPECIES GAME BIRD FARM WITH DETECTABLE AVIAN INLUENZA SUBTYPE H5N8 EXPOSURE

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INTRODUCTION

This presentation describes the difficult biosecurity situation encountered on a National Poultry Improvement Plan Avian Influenza H5/H7 Monitored game bird farm in which low path avian influenza subtype H5N8 was detected. The affected premises consisted of an approximately 30,000 bird conglomerate of pheasants, mallard ducks, quail, chukars, and pigeons, all raised within an area of approximately 2.3 ha (6 acres). The owner primarily marketed the birds to be used for bird dog trials. Many wild birds and feral pigeons occupied the farm along with free ranging chickens. Wild waterfowl had historically been seen intermingling with the captive ducks by entering through breaches in the netting. The owner had difficulty with a few pens of ill and dead pheasants.

Three dead juvenile pheasants were submitted to the Pennsylvania State University Veterinary Diagnostic Laboratory. Gross lesions suggested pasteurellosis or mycoplasmosis. *Pasteurella multocida* was cultured and *Mycoplasma gallisepticum* was identified by PCR. The primary diagnosis was avian cholera. Additionally, two birds were found virus-positive for low path avian influenza (AI) subtype H5N8. Findings were reported, the owner was contacted, and efforts were undertaken to address the detection of subtype H5 AI. Subsequent oropharyngeal swabs and serologic samples from gallinaceous species along with cloacal swabs from the ducks were collected. Pheasants were found to be actively shedding virus, and ducks had serologic evidence of H5N8 infection. Infected (3 km) and surveillance (10 km) zones were established. Testing of backyard poultry flocks within the 3 km (1.9 mi) zone yielded no detection of AI infection. A game bird operation belonging to a relative of the owner located 48 km (30 mi) away from the index farm was tested and no evidence of AI infection or exposure was found. All surveillance zone and contact premises remained negative on follow-up sampling.

Depopulation, cleaning, and disinfection procedures were commenced. Rodent baiting was aggressively undertaken. A total of 565 Norwegian rats (*Rattus norvegicus*) and 10 field mice (*Mus musculus*) were recovered. Typically, only about 30-35% of a total rodent population is recoverable; therefore, it is estimated that the baiting effort killed 1600 rodents (1).

After premises cleanup and surveillance efforts were completed, sixty (60) serologically-negative (to AI) sentinel pheasants were placed throughout the farm and sampled via oropharyngeal swabs weekly for four weeks for detection of AI shedding. All birds tested negative throughout the sampling period.

Reports of operational interactions, sampling procedures, diagnostic results, depopulation procedure, and description of interagency relations regarding this outbreak have been presented elsewhere (2,3,4).

CASE STUDY

The objective of this presentation is to describe the biosecurity challenges facing this multi-species game bird operation and actions taken. An assessment was made of geographic relationships of groups of birds, building locations, and traffic patterns of the affected property (Figure 1). After careful scrutiny of the premises and evaluation of the pen and species interrelationships, the following plan of action was undertaken.

Initial General Activities.

1. All domestic, and as many feral birds as possible, were removed.
2. All feed sources were removed, including open piles of corn and other ingredients, packages of unused feed, a hay stack, and feed left in hoppers and feeders.
3. Unused equipment, old boards, trash, and unused unsafe buildings were removed and hauled off premises.
4. Weeds, equipment, and debris at least two meters (7 ft) out from around buildings were removed.
5. Mice, rats, and other vermin were trapped and destroyed.

6. Pen netting was left up in most instances in order to discourage entrance of wild birds – particularly transient waterfowl. The netting was cleaned and disinfected as completely as possible or discarded in the case of the pens which were scraped by bulldozer.

7. Old litter, boggy areas, and dirt were scraped from barns, buildings, and pens and placed into dumpsters and taken to a landfill. Bare barns and buildings were heated to at least 32°C (90ºF) consistently for three days. This process took over two weeks to accomplish because of the size of barns, onset of cold weather, and propane equipment issues.

8. Trucks leaving the premises hauling possibly infected materials were power washed and sprayed with Virkon® S (DuPont).

Specific Cleanup Procedure.

1. Brooders and salvageable enclosures:
   a. Air tubes, feeders, waterers, and other loose equipment were removed and discarded (air tubes and wooden feeders) or cleaned, washed and disinfected for re-use after repopulation (waterers and metal feeders).
   b. Building heated to 32° to 38°C (90º to 100ºF) for at least 48 h.
   c. Buildings were spray washed and disinfected with a propionic acid/iodine mixture (Dyne-O-Might, Preserve International, Reno, NV).
   d. Buildings were heated back up to 32° to 38°C (90º to 100ºF) for at least 48 hr and dried out.
2. Flight pens and outside runs:
   a. Feeders and other washable equipment were removed.
   b. Ground cover, if present, was mowed down.
   c. Litter was scraped out of all pens.
   d. Standing water was drained or removed.
   e. Nets were kept up if possible to keep out wild birds and aid in sun disinfection of netting material (general premises rule).
   f. Pen areas were dried out and left vacant for at least 35 days.
3. Unoccupied, dilapidated, and/or unsalvageable construction:
   a. Torn down and hauled to landfill.

Follow-up Surveillance.

USDA Wildlife Services spent a week obtaining samples of the duck pond, rodents, pigeons, and other feral animals on the premises. Antibodies to influenza virus were detected via ELISA in six of seven (86%) specimens of the house mouse (*Mus musculus*). Subtypes were not characterized. No antibodies to influenza virus were detected in six *Rattus norvegicus*, one deer mouse (*Peromyscus maniculatus*), and one feral house cat. Antibodies to influenza virus were detected via AGID in six of 31 (19%) feral pigeon blood samples. Subsequent HI testing yielded the following HA subtypes: H1 (six pigeons), H4 (two pigeons), H7 (one pigeon), and H8 (one pigeon). All water samples were PCR negative for matrix influenza (i.e., there was no viral RNA detected) (5).

After all C&D was completed, AI-antibody-negative sentinel pheasants were placed (at least one to two birds) in each of the buildings and flight pen areas. Oropharyngeal swabs were collected on placement of birds, and at 7, 14, 21, and 28 d after introduction and tested by AI matrix rrt-PCR. All birds remained negative throughout the sampling period.

DISCUSSION

Cost of depopulation, cleanup, removal of debris, and surveillance activities was approximately $650,000. Incident length was 124 days, and approximately 2000 worker hours were spent on the project.

The biosecurity assessment presented unique challenges because of the complexity of species interactions, housing conditions, and widespread evidence of H5/N8 exposure/infection within the operation. A combination of approaches was necessary in order to get the premises in shape for the owner to resume business. Each building was assessed for suitability to C&D according to previously occupied species and their status of H5N8 shedding or infection, structural condition, clutter in and around the structure, and geographic location on the premises. All birds living on the premises were depopulated even though there was never evidence of AI infection in the captive pigeons, chukars, or quail. Interestingly, these species were housed in an area separate from the pheasant and duck brooders and flight pens (Figure 1). Feral pigeons, some roosting within buildings occupied by AI-infected pheasants or ducks, were considered potential vectors for AI spread and were summarily exterminated. Free ranging chickens were also depopulated. A concerted effort was made to reduce rodent population – especially mice, as these were shown to have detectable antibodies to influenza. From a purely biosecurity standpoint, rodent control would be especially important because of the strong probability of harboring *Pasteurella* and contributing to the spread of avian cholera, which was the primary diagnosis associated with morbidity and mortality.

Although not definitively proven, it is highly likely that the initial introduction of AI subtype H5N8 occurred through the intermingling of wild and captive ducks. The virus was then transferred to pheasants through close "fence-line" contact with the ducks, and possibly daily feeding and care activities. Feral pigeons

were shown to have been exposed to multiple HA subtypes, thereby acting as potential vectors for spread of AI. Although the HA subtypes were not characterized in the mice, the mere finding of such a high prevalence of influenza virus exposure in them indicates that they be considered as a high risk vector for AI spread.

It is hoped that findings made and actions taken in this incident serve as a guide in addressing future similar scenarios.

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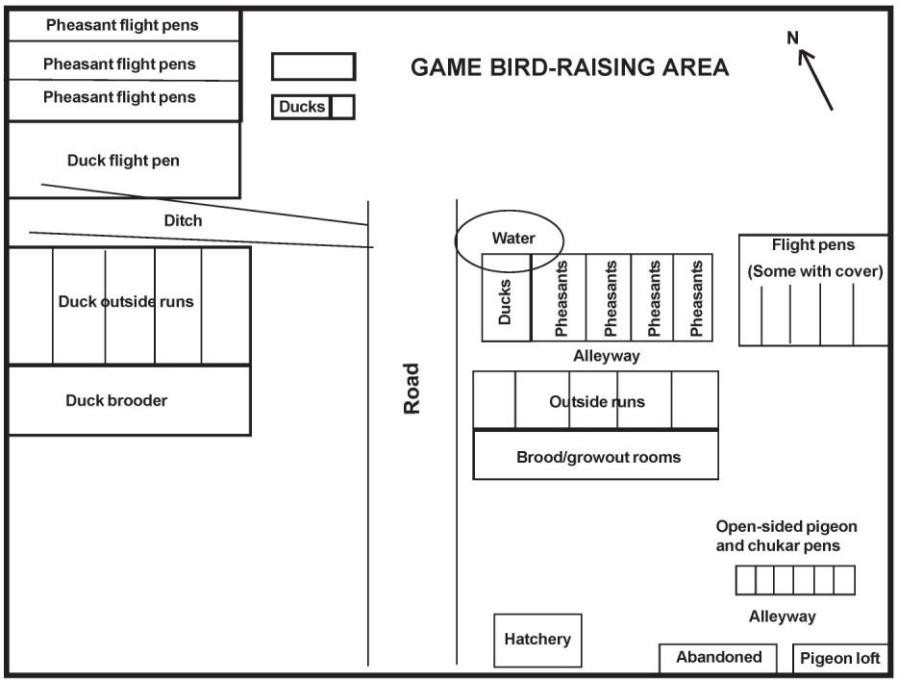
Figure 1. General farm layout illustrating relationship of different groups of birds to each other.



DEVELOPMENT OF A NOVEL PLENUM FLOOR FOR BROILERS TO REPLACE LITTER AND REDUCE HOUSE/ENVIRONMENT AMMONIA EMISSIONS

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Air emissions, particularly ammonia, are an emerging issue for all of animal agriculture. Agricultural operations are reported to be the largest source of ammonia emissions in the United States. Over the past several decades improved poultry production systems have contributed to significantly improved performance traits such as feed conversion, livability, meat yields, growth rates, and egg production, which provide economic, nutritious, and safe food.

In the United States, concentrated animal feeding operations (CAFOs) have greatly expanded. These operations produce large amounts of waste in relatively small areas. Broiler and turkey production are examples of CAFOs. These operations produce large amounts of waste in small areas.

Approximately nine billion broilers are raised in the United States that produce 25 billion pounds of manure. Approximately 250 million turkeys are raised that produce eight billion pounds of manure. The poultry industry in particular is challenged by regulators and environmental groups for its overall environmental footprint and impact on ecosystems. When ammonia nitrogen is released to the atmosphere, it reacts with acidic compounds where it can form haze and small airborne particles. It can contribute to fertilization of ecosystems causing acidification of the soil and eutrophication of water bodies. Ammonia itself is not considered by EPA to be a hazardous air pollutant. However, ammonia is a precursor in the formation of particulate matter of 2.5 microns or less ($PM_{2.5}$).

Poultry production systems must be further optimized to be energy and resource efficient with environmental sustainability. With potential future air emission restrictions, the United States Poultry Industry could lose its current competitive position in the world market. The U.S. currently produces 24% of the world's poultry meat.

In poultry production, birds are raised on a litter base on an earthen floor. The litter is used to absorb moisture from feces and other sources. This is used on multiple flocks before cleaning out. So the birds are raised on a mixture of decomposing feces and bedding or "built-up" litter. This litter is the major source of ammonia production and its management is a key factor affecting emissions rates.

Ammonia is a colorless, water soluble gas. Microbial degradation of uric acid in the litter is the primary source of ammonia formation and *Bacillus pasteurii* is one of the primary uricolytic bacteria that facilitate ammonia production. For optimum growth, the bacteria require a pH around 8.5. The decomposition process requires uric acid, water and oxygen to react giving off ammonia and carbon dioxide. It also requires the enzymes uricase and urease. The formation of ammonia can occur under aerobic or anaerobic conditions. Factors that contribute most to the formation of ammonia are temperature, moisture, pH and nitrogen content of the litter. The same conditions that favor the decomposition of uric acid favor the growth and proliferation of pathogenic organisms such as *Salmonella* spp. and *Escherichia coli.* High ammonia concentrations affect poultry performance which includes poor feed efficiency, blindness, and respiratory problems. There are also concerns for human health to workers in the poultry house environment. Timed limits are in place for human exposure. Concern has been expressed about ammonia on the basis of animal welfare as it affects the poultry house environment and paw quality. To date, no conclusive technology or best management practices (BMPs) emerge as a clear choice for the industry to use to mitigate ammonia emissions. Current approaches for managing ammonia include diet modification, modifying production environment (litter, acidification, moisture regime, litter composting, ventilation control), or modifying production structures (exhaust scrubbing, planted tree barriers).

With five years of engineering and testing, special flooring has been developed to replace the litter. The flooring is comprised of 18 in. x 18 in. (45.7 cm x 45.7 cm) interlocking squares made from injection molded polymers mounted on top of coned pegs to make a plenum. The squares have special engineered holes that allow moisture in the manure to be wicked away from the manure on the top of the flooring into the plenum below to be carried away by normal house ventilation.

In consequence, manure moisture on the top of the floor is extensively reduced and the pH of the manure is maintained below 8, which reduce ammonia production/volatization. The flooring system also seals the floor from the ground which prevents heat loss down and moisture migration up. With moisture and pH controlled of the manure base, a much better environment was produced in the broiler house. Other observed benefits were reduced dust levels ($PM_{2.5}$), lower coccidiosis cycling, no darkling beetles, and much improved broiler growth rate/FCR.

So eliminating litter with the innovative plenum flooring reduces ammonia production thus impacting the natural environment, poultry health/animal welfare, and broiler production parameters positively. Design and testing will be presented.

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ALTERNATIVE HOUSING SYSTEMS FOR LAYING HENS

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More than 10 years ago, on July 19, 1999, the Council of Agriculture of the European Union (EU) published minimum standards for the protection of laying hens. This European Council (EC) Directive bans the use of battery cages for laying hens by the year 2012.

The main points of the directive are:

- As of January 1, 2012, conventional cages will be prohibited.
- As of January 1, 2003, conventional cages must be equipped with suitable claw shorteners and provide at least 550 cm^2 (85 in^2) per hen.
- As of January 1, 2003, it is forbidden to install new or replacement conventional cages, all new or replacement cages must be enriched (furnished) ones - the standard requirements (750 cm^2/hen (116 in^2), access to a nest box, and 15 cm (6 in) perch space per hen) are applied to enriched cages (nest box, littered scratching area, and perch) as of January 1, 2002.
- As of January 1, 2002, new and replacement non-cage systems must fulfill the new standards. These standards include:
  - At least 1/3 of ground surface must be litter;
  - At least 15 cm (6 in) perch space must be provided per hen;
  - There should be no more than 120 hens per communal nest (1 m^2 (1.2 yd^2) per nest);
  - Stocking density should be no more than nine hens per m^2 (1.2 yd^2) of usable area.
  - As of January 1, 2007, all existing non-cage systems must fulfill the new standards.

Currently, the proportion of hens within the EU in non-cage systems to conventional cages varies among member states. Conventional cages are being phased out. Any new cages installed since 2003, and all cages from 2012, must be enriched (must provide a nest box, littered scratching area, and perch).

Presently within the EU about 60% of laying hens are housed in conventional cages. Some states (Germany, Austria) have banned conventional cages altogether, some other states still keep the majority of their laying hens in cages at this point. Consequently, the housing system for about 250 Mil. hens will have to be changed to alternative systems until 2012. In response to the new regulations, egg production has decreased within the EU. For the first time, egg supply has fallen below 100%. To meet current consumer demand, more eggs need to be imported from countries outside of the EU, which do not follow these regulations.

Worldwide, roughly 99% of laying hens are kept in conventional cage systems. Discussion processes have started in various other countries, amongst them the United States of America, where, in California, Proposition 2 (Prevention of Farm Animal Cruelty Act) was accepted by 63% of voters in 2008.

GEL DROPLETS DELIVERY OF COCCIDIOSIS VACCINE: FIELD RESULTS FROM SOUTH AFRICA

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SUMMARY

Oocyst counts of 430 fecal samples collected from chicken breeders and vaccinated by gel droplets delivery are reported here. They were collected from March 2008 to June 2010 from eight rearing farms belonging to an integrator that produces over 2.5 million breeders a year in South Africa. Some of these samples were combined to give final counts totaling 339, 100, 108, 73, and 58 from birds one week, two weeks, three weeks, and four weeks post vaccination (PV) respectively. Fifty six percent of these were vaccinated in the hatchery and the rest in the chick trays on the barn floor before dumping. Oocyst counts averaged from less than 2000 per gram of feces and peaked at over 70,000 at three weeks PV before dropping to 3500 oocysts at four weeks PV. A little more than half (53%) were positive when vaccinated on the barn floor. But, at least 74% of the fecal samples were positive seven d PV when birds were vaccinated in the hatchery, similar to the 75% uptake, or three of four pens of 50 birds each, found in a pen trial previously conducted in Georgia.

INTRODUCTION

Exposure to a coccidiosis vaccine with the oocysts suspended in edible gums was first reported 25 years ago (3). This was later recognized by Shirley and Long (6) as a means to achieve uniform uptakes. Several methods for delivering coccidiosis vaccines have also been tried for a few years before the adoption of water spray (2) and in our case, gel spray or droplets. These methods include spray on feed; spray on eyes; droplets manually applied to eyes or beaks; gel pucks; water spray; and gel droplets. Whether the delivery is carried out in the hatchery or on barn floor, there is one thing in common: they are all being applied, with rare exception, only once because of cost. However, even with just one application we know that protective immunity against coccidiosis outbreaks can be achieved by the recycling oocysts (4). But relying on recycled oocysts for protective immunity sometimes can be more of a problem than a solution because the numbers produced by different species have been shown to range from ten thousand to tens and hundreds of thousands from each ingested oocyst (1).Therefore knowing how the coccidiosis vaccines behave in the field may help to understand why coccidiosis vaccination may not work some of the time as manifested mostly by related bacterial enteritis. Here, a composite profile of oocysts counts from 430 litter/fecal samples collected over 100 visits from March 2008 to June 2010 from eight rearing farms belonging to one integrator in South Africa rearing over 2.5 million broiler breeders per annum, may be a useful representation of how coccidiosis vaccines behave in the field.

MATERIALS AND METHODS

Vaccine and vaccination. All chicken breeder hatchlings were vaccinated either in the hatchery or in the chick trays on the barn floor just before they were dumped. They were all vaccinated with Immucox® (Vetech Laboratories Inc.) with about 60% vaccinated in the hatchery and the rest (40%) on the barn floor all with gel droplets delivery method (5).

Litter/feces collections and processing. Litter/feces samples were collected at 7, 14, 21, and 28 d post vaccination (PV). An average of four samples of various weights of litter/feces was collected from each visit. They were submitted to Deltamune Laboratory, an independent laboratory, for processing and oocyst counts. The samples, if not processed right away, were kept at 4°C for not more than one week. Each sample was processed in a 100 g lot or adjusted proportionally before the homogenization in a table top blender. The homogenates were placed on a shaker and shaken overnight to loosen oocysts still hanging in the litter. They were then filtered and isolated by centrifugation. The final sediments before counting were floated in a saturated sugar solution. Oocysts per g were determined by counting using the Modified McMaster Method.

RESULTS AND DISCUSION

At seven d PV oocysts were recoverable from 74.1% (43/58) litter/feces samples collected from broiler breeders vaccinated in the hatchery (Figure 1) with an average oocyst count of 814/g in comparison, 52.5% (21/40) of the samples collected from breeders sprayed on the barn floor having an average count of

1,735 oocysts/g. Interestingly, in a pen trial carried out in Georgia recently, oocysts found in fecal samples were in about the same proportion seven d post vaccination (PV). Three of four pens were positive with oocysts numbering 67 to 400 per g from about 10 g of fecal samples collected.

At 14 d post vaccination there was a jump from under 2,000 oocysts average to almost 39,303 and 61,160 for hatchery and barn application respectively, an increase of 20 times or more and with oocysts recoverable from over 96.6% (56/58) and 94.7% (36/38) respectively from all samples collected. Although there were further increases at 21 d PV, the increases on average were of smaller magnitude or to 77,199 and 71,852 respectively. Here when the hatchery application started to decrease to 88.1% (37/42) on average, the barn application was still on the increase to 96.5% (30/31). At 28 d PV, although oocyst was still found in 89.8% of all samples collected the numbers of oocysts have declined to 19,714 and 3,491 from hatchery and barn application respectively.

This profile of oocyst counts with the proportions of positive fecal samples collected suggests the following. As long as the proportion of uptakes observed in seven d PV is 50% (52% and 74%) or more, the entire flocks will be positive in the second cycle (95% to 97%). At the same time, it helps if the numbers of oocysts available for recycling are not exceedingly large. Then the first recycling becomes a stimulation of a second inoculation. From this point on the proportion of naïve birds will be negligible to none and therefore the flock can now ingest the oocysts that have increased by 20 times or more with impunity. The reverse should also be true: if the proportion of uptakes is small or much less than 50%, or the number of oocysts available is too high at seven d PV, there will still be some naïve birds available if some of them have not been damaged already by the high doses of excreted oocysts around seven d PV. Most likely it is this small proportion of birds that are still naïve or remain not fully protected at, or after, 14 d PV that are the fodder for the bacterial enteritis.

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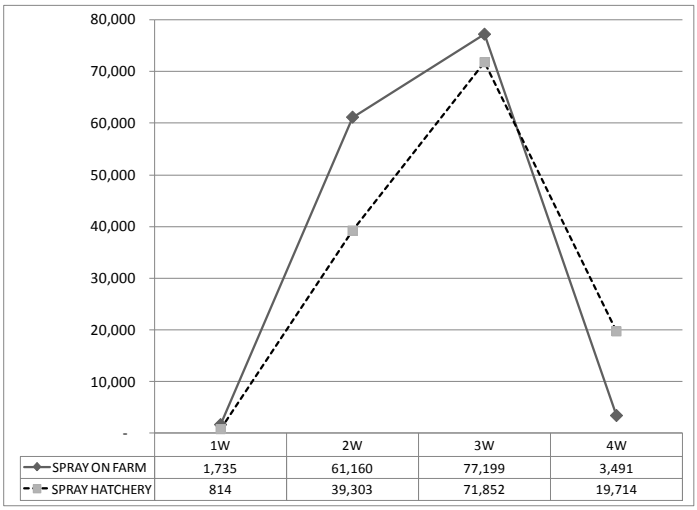
Figure 1. Profile of oocysts counts of 430 samples collected from March 2008 to June 2010 from eight broiler breeder farms in South Africa at the four weekly intervals after coccidiosis vaccination.



GEL DROPLETS VACCINATION METHOD FOR THE DELIVERY OF NEWCASTLE DISEASE VACCINE IN THE BARN

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SUMMARY

Gel droplets vaccination as a method for the delivery of coccidiosis vaccine in hatchery and barns has been used successfully for the last few years. Experimental use of delivering infectious bursal disease (IBD) and *Salmonella* vaccines using gel droplets again showed that this also can be extended to (header, and control chicks by water through bell-shaped drinkers. Enzyme linked immunosorbent assay (ELISA) test on serum samples collected three weeks after vaccination showed that 9/10 of chickens vaccinated by gel droplets spray have antibody to Newcastle disease virus (NDV) compared to 8/9 chickens by drinking water. The mean ELISA titers were 2305 and 2946 in chickens vaccinated by gel droplets and in bell-shaped drinker respectively. These results show that NDV vaccine can also be delivered by gel droplets method.

INTRODUCTION

Newcastle disease (ND) is a highly contagious disease affecting chickens and other avian species such as turkey and pigeons (1). The ND virus (NDV) is widely distributed and the occurrence of pathotypes is different from area to area, but the lentogenic strains of NDV are worldwide in distribution (8). Beside biosecurity practices; vaccination is one of the most common practices to control NDV in most parts of the world. Live NDVs of low virulence or of moderate virulence as well as inactivated vaccines are used for vaccination of poultry. NDV live vaccines may be applied individually by the conjunctival or intranasal application or the beak dipping. More commonly, live NDV vaccines are administered in drinking water or delivered as a coarse spray (1,8). In drinking water application of NDV vaccines, the virus may be inactivated by disinfectant in the water, and sometimes even by the type of pipes or vessels used (1). The laborious steps of water vaccination likely add costs to the application of vaccines (2). The use of the gel droplets spray method may make these laborious steps of water vaccination unnecessary (5,6,7). This study is an attempt to show that gel droplets delivery system can be used successfully to vaccinate chickens against ND.

MATERIALS AND METHODS

Experimental chickens. All the chicks here were hatched in our laboratory from SPF eggs obtained from Sunrise Farm Inc. Hatchlings were placed in single-use cardboard boxes and housed in a disinfected isolated quarter. Feed and water were supplied *ad libitum.* At day nine 25 chicks/experiment were divided into three groups. Two groups of 10 chickens each were either vaccinated by water or by gel. Five chicks served as unvaccinated controls.

The gel diluents. The gel-sprayed vaccines were delivered with 1.3% of the 60/40 gel diluent of Vetech Laboratories Inc. and 0.1% of xanthan gum was added (Lee, USA Patent pending). Red or green food color was added to the mixture as an indicator for vaccine take.

NDV vaccination. The NDV vaccine stabilizer was prepared by suspending 2.5 g/L of skim milk powder in distilled water (3). The lyophilized 1000 doses NDV vaccine (B1 type, Fort Dodge) was first dissolved in 4 mL of the vaccine stabilizer then added to gel diluent or to the water, both containing 2.5% skim milk powder. In the first experiment the compatibility of the NDV vaccine with the gel diluent was tested. The NDV vaccine was mixed with the gel diluent and compared to mixing in water. The chickens were divided randomly into three groups: NDV Gel vaccinated, NDV water vaccinated, and unvaccinated controls. Each group of chickens were kept in a single-use cardboard box. After dissolving the lyophilized NDV, 0.25 mL was added to either 625 mL of gel diluent or water. Water was withdrawn for 2.5 h before vaccinating the chickens. Newcastle disease virus vaccine suspended in gel or in water was given in bell-shaped drinkers, at the rate of 10 mL/chicken. In the second experiment, 3 mL (750 doses) were added to 375 mL of the gel diluent and about 0.5 mL/chicken was used for the droplets spraying. The remaining of the reconstituted vaccine (250 doses) was added to 2.5 L of the stabilizer, and 10 mL/chicken was used for the water vaccination and given in a bell-shaped drinker. For gel droplets vaccination, chickens were placed in a cardboard disposable box and sprayed from the top, and left to preen the sprayed vaccine. The reconstituted ND vaccine in the gel was delivered using hand held sprayer attached to it a special multi-opening device

(header). Water was withdrawn from both groups for about two h before vaccination.

Blood sampling. Blood samples were withdrawn from jugular vein using 1 mL disposable syringes and sera were separated by centrifugation. Samples were collected from chicks at 3, 14, and 21 days post vaccination. Sera were stored frozen at -20°C, and then submitted to the Animal Health Laboratory, University of Guelph, to test for the presence of antibody to NDV using ELISA tests.

RESULTS

NDV vaccination. The NDV vaccine was well consumed by the chickens whether reconstituted in water or in gel when placed in the bell-shaped drinkers. When the vaccine in gel was sprayed on the chickens, the droplets were preened and most droplets were picked up within minutes.

Antibody response to NDV vaccination. In the first experiment, the mean ELISA titers of chickens vaccinated in water and gel diluent are shown in Table 1. The antibody response of chickens was low in both groups whether they are vaccinated in water or in gel diluent two weeks post vaccination (Table 1). The mean antibody titers was higher in both vaccinated groups, at three weeks post vaccination but the group that received the vaccine through gel diluent appear to have higher mean ELISA titers, and 9/10 chickens responded to vaccination compared to 8/9 of the chickens that were vaccinated in water (Table 1). In Experiment 2 the mean antibody titer of the group vaccinated by water in bell-shaped drinkers were generally higher than those titers of chickens vaccinated by gel droplets method at two and three weeks after vaccination (Table 1); however, more chickens have detectable antibodies in gel droplets vaccinated than water vaccinated chickens at two and three weeks after vaccination (Table 1). At two weeks post vaccination 8/10 and 6/10 chickens were positive in gel droplets method and water vaccination respectively. At three weeks post vaccination 9/10 and 8/9 were positive in gel droplets method and water vaccination respectively (Table 1).

DISCUSSION

Adding this gel droplets method for delivering NDV vaccine to replace the more tedious procedure by drinking water should be an advantage. This alternative method helps the live vaccine to avoid chlorine before and after vaccination as well as cutting short the stress of water deprivation to the birds (2,3,4). In this study, water withdrawal was not necessary in Experiment two for the gel spray method but was practiced to treat vaccinated groups equally. In this study, it was shown that the mixing of NDV vaccine with the gel diluent did not interfere with the immune response of the vaccinated chickens when given orally. In fact, the mean ELISA antibody titer in Experiment 1 was slightly higher in chickens than of the positive controls three weeks post vaccination (Table1). In the second experiment more chickens responded to NDV vaccine in the group that was vaccinated by gel droplets than those vaccinated by water at two and three weeks post vaccination, but the mean titer was lower (Table 1). The reason for that could be the lower amount of the vaccine that consumed after spraying compared to the longer exposure of the entire volume consumed from the bell-shaped drinker. This explanation is in agreement with the result of Experiment 1: when the chickens had the chance to consume larger volume from the vaccine, better response was obtained (Table 1). Perhaps increasing the volume to 0.6 mL/chicken or higher may give better response from the gel droplets vaccinated chickens (9).

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Table 1. Mean ELISA antibodies titers of chickens vaccinated against Newcastle Disease, delivered by gel droplets spray method, or in bell-shaped drinkers either in gel diluent or in water.

Vaccine delivery	3 days PV[A]	Week 2 PV	Week 3 PV
Experiment 1			
Water vaccination in Bell-shaped drinkers	-	179[B] (1/10[C])	1565 (8/10)
Gel vaccination in Bell-shaped drinkers	-	155 (1/10)	1609 (9/10)
Unvaccinated	39 (0/5)	86 (0/5)	362(1/5)
Experiment 2			
Water vaccination in Bell-shaped drinkers	1 (0/3)	2141(6/10)	2946 (8/9)
Gel droplets Spray	1 (0/3)	788 (8/10)	2305 (9/10)
Unvaccinated	26 (0/3)	122 (1/5)	159 (1/5)

[A]PV= post vaccination.
[B]Mean ELISA titers.
[C]Number positive/total tested.

EFFICACY OF VAXXITEK™ AND A COMMERCIALLY AVAILABLE OIL-EMULSION NDV VACCINE ADMINISTRATED SIMULTANEOUSLY BY THE SUBCUTANEOUS ROUTE USING THE ACCUVAC™ TWINSHOT OR THE ONE-SHOT MACHINES TO VACCINATE DAY-OLD COMMERCIAL BROILERS

Rafael J. Fernandez, Julio Cruz, Francisco Rojo, Hector Garcia, Jerome Baudon, and Nikki Pritchard

Merial Select, Inc. Gainesville, GA

Two hundred seventy (270) one-day-old commercial broilers, Harrison Poultry Flock 8-2, were divided into two different vaccination treatments groups. The birds in Groups 3 and 4, the non-vaccinated, challenged and non-challenged controls, respectively, were placed into their designated units before the vaccinations. The remaining birds were then vaccinated with VAXXITEK™ and the NDV oil-emulsion with either the ACCUVAC™ Twinshot or the Twinshot-Plus Machines. The birds were vaccinated subcutaneously (SQ) with 0.2 mL per chick with VAXXITEK or with 0.1 mL per chick with the NDV oil-emulsion. After the SQ vaccinations, all the birds in Groups 1 and 2 were vaccinated IO with NDV La Sota, 0.03 mL per dose/bird. On study day five, 90 birds in Groups 1-3 were challenged with the vvMDV RB1B, by the intraperitoneal (IP) route, 0.2 mL per bird. The birds were observed daily for 45 days post-challenge for any unfavorable reactions to the challenge, particularly death or depression.

On study day 21, 10 birds in Groups 1-2 and five birds in Groups 3-4 were wing-bled and sera was collected for NDV hemagglutination inhibition (HI) antibody testing and IBDV ELISA evaluations. Fifty birds in Groups 1-3 were challenged with NDV GB Texas, 0.03 mL/IO dose. In addition, another 50 birds

in Groups 1-3 were challenged IO with IBDV STC strain, and another 50 with IBDV Variant "E". The birds 1-3 challenged with NDV GB Texas were observed for 13 days for severe clinical signs of NDV, such as but not limited to, extreme nervousness, respiratory distress, nervous signs or death. All remaining birds were humanely euthanized at the end of the observation period. On study day 25, all the IBDV STC challenged birds in Groups 1-3 were bled for IBDV ELISA evaluation and humanely euthanized. Bursas were observed for IBD lesions, collected and placed in buffered formalin for further histopathological evaluation. On study day 28, all the IBDV variant "E" challenged birds in Groups 1-3 and the ten birds in the non-challenged control Group 4 were bled for IBDV ELISA evaluation and humanely euthanized. Bursas were observed for IBD lesions, collected, weighed and placed in buffered formalin for further histopathological evaluation. On study day 50, the birds were humanely euthanized and necropsied to examine for gross lesions associated with Marek's disease.

(Results will be presented.)

USE OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATION IN BROILER PROGENY AS A TOOL TO EVALUATE BREEDER INFECTIOUS BURSAL DISEASE VACCINATION PROGRAMS

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INTRODUCTION

Negative effects of infectious bursal disease (IBD) on the immune system of broilers can be effectively controlled by the appropriate maternal antibody profiles. However the IBD virus is not entirely stable and will experience subtle shifts in viral antigens and pathotype. These shifts over time may not be covered by the maternal antibody profile induced by the breeder vaccination program which can result in broiler flock immune system compromise. This in turn can cause significant economic losses due to poor performance and increased mortality.

Evaluating broiler breeder IBD vaccination programs using bursas collected from broiler progeny is a common industry practice. Historically this evaluation has been done by using bursa to body weight ratios, histology, histology with limited virus isolations and PCR. These protocols tend to be somewhat limited because they fail to effectively address the timing of IBD virus infection and typically have minimal relevance to the integrity of the bird's immune system. Most importantly they do not address changes in IBD virus antigenic configuration.

Since maternal antibody effectively neutralizes IBD virus in broiler progeny when maternal antibody is produced against the homologous antigenic and pathotype field virus, IBD virus isolations from broiler progeny gives a reliable evaluation of the degree of protection afforded by maternal antibody. The purpose of this paper is to describe the procedures utilized and results from using this approach to evaluate and adjust broiler breeder IBD vaccination programs to ensure protection against relevant field IBD virus(es).

The procedures followed for this approach are outlined below in the instructions given to companies interested in evaluating their IBD breeder vaccination programs.

SURVEILLANCE AND CHARACTERIZATION

Protocol for IBDV surveillance in broiler flocks. Bursae of Fabricius should be collected from six birds in each of 30 broiler flocks per complex. This assumes the size of the complex allows the collection of this many samples from the preferred age groups. The broiler flocks selected for sampling should be derived, in as much as possible, from farms ranked in the lower third of producers in terms of performance and be between the ages of 12 to 24 d of age. Birds from flocks meeting the aforementioned criteria should be submitted alive to a designated location (diagnostic laboratory, etc.) for necropsy and bursa collection. The bursae of Fabricius are collected individually from each farm and the individual bursae (six per farm) placed in each of six wells of a 12 well plate (two farms per plate). A set of clean instruments should be used for each farm. After each use the instruments

should be cleaned with alcohol swabs provided and placed in a beaker of alcohol for five minutes. The plates containing the bursa samples should be labeled with company, complex, farm name, flock age, and collection date. The instruments and 12 well sampling plates can be provided by AviServe LLC. The bursae should be placed on cold packs during collection and shipped overnight to AviServe LLC in a provided shipping container. If they can't be shipped immediately post collection they should be frozen and shipped when feasible.

Upon receipt the bursae are evaluated for size and appearance and prepared for virus isolation and RT-PCR. IBDV isolates are then assessed for antigenic differences. If they appear to be different antigenically they can then be pathotyped *in vivo* and selected areas of the viral genome sequenced.

Information provided by IBDV surveillance. Surveys provide data on the relative degree of susceptibility of broilers in different growing areas. This is accomplished by determining both the time (age) of field infection and number of flocks infected. This information can be useful for comparing IBDV susceptibility within and among different integrators and can be used to evaluate the relative protection provided progeny by different breeder vaccination programs.

Viruses collected can be characterized, which provides potentially important information on the evolutionary status of IBDV. Within particular companies and/or geographic areas carefully selected isolates can be used for progeny challenge assessments, evaluations of available vaccines via direct challenge, and for the preparation of autogenous vaccines and/or commercially licensed products.

RESULTS AND DISCUSSION

Table 1. Company A presently uses a commercial program that includes low bursa derived antigen sources and reports significant seasonal bird health and condemnation issues

No. of IBD Isolates/total no. flocks samples %	**No. IBD Isolates/Total % Company A**		
	13-17 days	20-21 days	23-25 days
19/31 61%	5/15 33%	5/6 83%	9/10 90%

Table 2. Company B. was using a commercial program that used only high bursa derived antigen sources and reported minimum bird health and condemnation issues in 2006. Company B selected virus antigens from the population that broke through the commercial antigen profile and produced an autogenous vaccine.

No. of IBD Isolates/total no. flocks samples %	**No. IBD Isolates/Total % Company B 2006**	
	12-20 days	21-29 days
66/148 44%	23/148 15%	43/148 29%

Table 3. Company B results using high bursa derived commercial antigens with a high bursa derived autogenous vaccine.

No. of IBD Isolates/total no. flocks samples %	**No. IBD Isolates/Total % Company B 2010**	
	12-20 days	21-29 days
34/165 20%	1/165 <1%	33/165 20%

VACCINATION OR MEDICATION FOR THE CONTROL/ ERADICATION OF PATHOGENIC AVIAN MYCOPLASMAS

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Pathogenic mycoplasmas associated with management deficiencies and concurrent infections induce chronic respiratory disease (CRD) that is still an important health problem for commercial poultry all over the world. *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) infections interact with other pathogens like Newcastle disease, infectious bronchitis, avian influenza viruses, and *Escherichia coli* producing CRD which appears to be more difficult to control due to complications with immunosuppressive diseases and mycotoxins in feed; therefore, is important to consider different approaches for the control or eradication of these pathogens.

Control of pathogenic avian mycoplasmas consists of one of three general approaches: Maintaining flocks free of infection, medication, or vaccination. In order to maintain flocks free of pathogenic mycoplasmas, replacements from mycoplasma free sources must be kept in a single age, all-in all-out management system. Good biosecurity and an effective monitoring system are necessary aspects of this program. Medication can be very useful in preventing clinical signs and lesions, as well as economic losses, but cannot be used to eliminate infection from a flock and is therefore not a satisfactory long-term solution. Vaccination against *Mycoplasma gallisepticum* (MG) or *M. synoviae* (MS) can be a useful long-term solution in situations where maintaining flocks free of infection is not feasible, especially on multi-age commercial egg production sites, but is also used in some areas in multiple age breeding stock farms.

An effective biosecurity program should be adequate for maintaining flocks free of mycoplasma infection. However, there are now large concentrations of poultry in small geographic areas thereby increasing the probability of exposure, especially when there are lapses in biosecurity. Control strategies commonly used for the control of pathogenic avian mycoplasmas within the poultry industry include intense biosecurity and biosurveillance via serologic testing, MG isolation, and DNA-based detection methods.

Furthermore, complications toward MG control include the organism's ability to transmit both vertically and horizontally and survive outside the host and the lack of rapid and specific means of detection that differentiates field and vaccine strains.

Choices include inactivated oil-emulsion bacterins, live vaccines, or a recombinant live poxvirus vaccine containing and expressing key protective antigens. A temperature sensitive mutant of Australian field strains of MG and MS have been shown to be safe and efficacious for chickens and have proven to be beneficial in several areas of the world that have problems with virulent strains. Within the layer industry, live attenuated MG vaccines have been approved and are widely used to minimize the effect of infections by virulent MG.

Medication. Since mycoplasmas lack a cell wall, they are resistant to β-lactamic antibiotics such as penicillins or cephalosporins. However, they tend to be sensitive to macrolides, tetracyclines, fluoroquinolones, and others. There are also numerous reports of efficacy testing of various antibiotics in infection models and in naturally infected birds. Antibiotic resistance has been reported. Antibiotic medication has been used to reduce egg transmission and to improve egg production in MG-infected commercial layers. Dipping of hatching eggs in antibiotic solution or injection of individual eggs has been used to reduce or eliminate egg transmission of MG and MS. Currently tylosin or tetracyclines are the most commonly used products for reduction of egg transmission or for prophylactic treatment to prevent respiratory disease in broilers or commercial turkeys. Highly effective products, such as enrofloxacin or tilmicosin, are not approved for use in poultry in the United States. A typical treatment program in infected breeding stock may consist of continuous medication in the feed or treatment for five to seven days each month. Treatment may reduce the populations of mycoplasmas in the respiratory tract, thus potentially reducing the risk of spread to neighboring flocks. Medication of naturally infected birds with enrofloxacin was highly effective in reducing or eliminating upper respiratory infection and

egg transmission in hens challenged with MG. There is one report of apparent eradication of MS from a flock after intensive treatment for control of *E. coli*.

Nevertheless, even though antibiotic medication can be an effective tool for the reduction of egg transmission, clinical signs, and lesions, medication cannot be depended upon to completely eliminate infection from a flock and continuous use may result in the development of resistance. Antibiotic medication can be effective and useful in preventing economic losses associated with avian mycoplasmas infections, but it should not be considered to be a long-term solution.

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PERSISTENCE OF RECOMBINANT FOWL POX VIRUSES IN CHICKEN TISSUES FOLLOWING VACCINATION AND THE LOCAL IMMUNE RESPONSE AT THE SITE OF INOCULATION

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SUMMARY

Fowl pox virus (FPV) has been under development as a recombinant vaccine vector for 20 years. To date, surprisingly, very few data exist on the persistence of fowl pox vaccine virus in chicken tissues, or the immune cells that respond to the vaccination at the site of inoculation. Although both humoral and cellular mediated immunity (CMI) play a part in overall immunity against FPV, little is known regarding the cell-mediated immune responses to FPV infection.

The main aim of this study was to measure persistence of recombinant fowl pox vaccine virus in skin tissues following vaccination. The recombinant FPVs did not persist for long. Virus was detected in skin tissue after vaccination at very high concentrations two days post-vaccination (dpv), to a lesser degree at four dpv and was almost cleared from six dpv. We also investigated the kinetics of response of immune cells (macrophages, B cells, $CD4^+$ and $CD8^+$ T cells) in infiltrating the site of vaccination.

INTRODUCTION

While attenuated fowl pox virus (FPV) strains are widely used for vaccination of chickens and turkeys for prevention of fowl pox infection, recombinant FPV expressing various foreign genes have been evaluated for their ability to offer protection against various diseases in poultry as well as in mammals. FPV has been used as a vector for quite a long time. To date, surprisingly, very little data exist on the persistence of fowl pox vaccines in chicken tissues, or what kind of immune cells respond to the vaccination at the site of inoculation. Although both humoral and cellular mediated immunity (CMI) play a part in overall immunity against FPV, little is known regarding the

cell-mediated immune responses to FPV infection. It was showed that FPV infection causes a lymphoproliferative response in chickens and that the majority of proliferating T cells, largely $CD8^+$, were virus antigen-specific (1).

The main aim of this paper was to measure persistence of the recombinant fowl pox vaccine in the skin tissues of chickens following vaccination.

The skin has an important role in innate and adaptive immunity. It is well known that most micro-organisms that come into contact with the skin barrier do not penetrate it. In addition, the blood and lymphatic networks act as highways on which immune cells travel to get to and from their sites of action. The skin houses many immunological cells. Since the primary function of skin is to provide an effective barrier against the outside world, it is likely that a rapid and efficient host defence system exists and can be triggered once the barrier has been broken.

MATERIALS AND METHODS

Chickens. Rhode Island Red (RIR) chicks were obtained from an unvaccinated flock maintained in isolation accommodation at the Institute for Animal Health, Compton, UK. The experiments met with local ethical guidelines as well as those of the UK Home Office.

Viruses. Fowl pox virus FP9 derivative fpIBD1 (2), expressing most of the IBDV F52/70 VP2 protein as a β-galactosidase fusion protein under the control of the Vaccinia virus p7.5 early/late promoter, from the BglII insertion site in ORF FPV002, was from laboratory stocks. fpIBD1 was grown on chicken embryo fibroblast (CEF) cells in the presence of 1X 199 medium (Sigma).

fpIBD1 mutants carrying only deleted forms of the putative IL-18 binding protein (IL-18bp) genes (fpIBD1Δ073 and fpIBD1Δ214) and other mutants containing chicken IL-18 (ChIL-18) (fpIBD1::ChIL-18, fpIBD1Δ073::ChIL-18 and fpIBD1Δ214::ChIL-18) were used too.

Experimental design. One hundred and twelve chicks at one week of age were divided into eight groups, 14 birds in each group as follows:

Group 1 were inoculated with 50 μL PBSa per bird (negative control).

Group 2 were inoculated with 250 μg/mL PHA per bird (positive control).

Group 3 were inoculated with fpIBD1.

Group 4 were inoculated with fpIBD1Δ073.

Group 5 were inoculated with fpIBD1Δ214.

Group 6 were inoculated with fpIBD1::ChIL-18.

Group 7 were inoculated with fpIBD1Δ073::ChIL -18.

Group 8 were inoculated with fpIBD1Δ214::ChIL -18.

The vaccination dose at one week of age with these six viral vaccines was 10^7 pfu in a 50 μL volume. The inoculum was placed on both wing-webs of each bird and the skin punctured 30 times over an area of 2 mm^2 with a 21-gauge hypodermic needle. Two birds from each group were killed each time point by cervical dislocation and skin samples from the site of inoculation were taken. The first sample was taken two days post-inoculation and sampling was then carried out every two days till 14 days post-inoculation. Two skin samples from each bird (both wing-webs) were taken, one of the wing-webs was taken for real-time quantitative PCR to measure viral load, and the other for immunohistochemical staining (for FPV, macrophages, $CD4^+$ T cells, $CD8^+$ T cells and B cells), to estimate the relative number of positively staining cells at the site of inoculation using Image-Pro® Plus soft-ware version 4.0. Two skin sections (from two birds) and three fields of view per skin section were used at each time-point for image analysis. This is a semi-quantity measurement, as it gives a percentage of the whole microscopic field (per area) that stained positive.

Sample processing.

DNA extraction. Up to 25 mg skin were cut into small pieces, placed in a 1.5 mL micro-centrifuge tube. DNeasy Tissue Kits from QIAGEN was used for rapid isolation of total DNA following the manufacturer's instructions.

Frozen sections for immunohistochemical staining. Each skin sample was put on a 2.5 cm^2 cork tile and covered with Tissue-Tek® O.C.T™ Compound. The samples were then snap-frozen in a dry-ice/iso-pentane bath and transferred to liquid nitrogen. Frozen blocks were then removed from the liquid nitrogen, wrapped in aluminium foil and stored at -70°C. Sections (6-8 μm) were then cut from these blocks for immunohistochemistry staining using a cryostat, picked up onto glass slides, then fixed in acetone for 10 min and air-dried. Staining was then carried out using a Vectastain® ABC αmouse IgG HPR staining kit (Vector Laboratories, Burlingame, CA, USA), following the manufacturer's instructions. The monoclonal antibodies used were DF6 (3) for FPV, KUL01 (4) for macrophages, AV14 (5) for CD8, AV29 [Fred Davison, IAH] for CD4 and AV20 (6) for B cells.

Real-time quantitative PCR. The fluorescently labelled probes were labelled with the reporter dye 5-carboxyfluoroscein (FAM) at the 5′ end and the quencher N,N,N',N'–tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end. Specific primers were designed to closely flank the probe. Primers and probes sequences are given in Table 1.

Amplification and detection of specific products were undertaken using the ABI PRISM™ 7700 Sequence Detection System. Quantification was based on the increased fluorescence detected by the ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems) due to hydrolysis of the target-specific probes by the 5′ nuclease activity of the *rTth* DNA polymerase during PCR amplification. Results are expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye (ΔR_n) passes a significance threshold.

Construction of standard curves for quantitative PCR assays. Standard curves for the DNA-specific reactions were generated from real-time PCR reactions on serial $\log_{10}$ dilutions of DNA standards (usually plasmid DNA containing same DNA template), and were included in every experiment. Dilutions of 10^{-1} to 10^{-9} were made in DEPC-H_2O. Regression analysis of the mean values for the $\log_{10}$ diluted DNA plasmid was used to generate a standard curve. Briefly, by knowing the plasmid size, and concentration, the number of plasmid copies can be easily quantified. The number of sample DNA copies could then be calculated from the sample C_t values. For example:

Plasmid pCI-neo::VP2 was used as a standard. The plasmid is 7105 bp in size (5474 bp pCI-neo + 1631 bp VP2), and the concentration of the DNA was 1400 µg/mL.

The average molecular weight of DNA is 660. Therefore, 7105 X 660 = 4689300 g which is the number of grams in one mole of plasmid. So, number of grams in 1 mL/number of grams in 1 mole = number of moles of plasmid per mL of DNA. (1400 X 10^{-6})/4689300 = 2.985 X 10^{-10} moles/mL. Avogadro's number is the number of molecules in 1 mole = 6.022 X 10^{23}. Therefore we have (2.985 X 10^{-10}) X (6.022 X 10^{23}) molecules per mL DNA = 1.798 X 10^{14}.

Five µL DNA was used in each Taqman reaction, therefore [(1.798 X 10^{14}) / (1000)] X 5 = 8.99 X 10^{11} molecules in 5 µL of undiluted DNA. (8.99 X 10^{11}) X dilution factor gives number of molecules in the reaction. This is graphed against C_t values for dilution. The equation of the plasmid dilution curve regression line **[y = -1.4925Ln(x) + 38.973]** was used to estimate the number of viral copies per sample:

x = exp((y-38.973)/-1.4925), where x is VP2.

As there were two copies of VP2 inserted into fpIBD1 in the ITRs (2), the resulting values were divided by two.

RESULTS

fpIBD1 and altered fpIBD1 persistence in tissue following vaccination.

Using immunohistochemical staining. Skin sections from the site of vaccination were stained using a specific monoclonal antibody (DF6) against FPV (3).

All six viruses (fpIBD1, fpIBD1Δ073, fpIBD1Δ214, fpIBD1::IL-18, fpIBD1Δ073::IL-18 and fpIBD1Δ214::IL-18) were detected at 2 dpv and to a lower degree at 4 dpv. No virus was detected from 6 dpv onwards (Fig. 1.1, an example of staining patterns following fpIBD1 vaccination).

Using real-time quantitative PCR. DNA was extracted from the skin at the site of vaccination. Real-time quantitative PCR was carried out to detect virus in the skin tissue. A very high number of viral copies was detected at 2 dpv and 4 dpv, whereas very low viral titers were detected from 6 dpv onwards.

Influence of immune cells to the site of vaccination.

Macrophages. Skin sections were stained using the monoclonal antibody KUL01 (4) (Fig. 1.2). The pattern of macrophage infiltration at the site of vaccination in the skin is similar to that of the virus, in that high numbers were seen at 2 dpv and 4 dpv, and these dropped from 6 dpv onwards.

$CD4^+$ T cells. Skin sections were stained with the monoclonal antibody AV29, which recognizes $CD4^+$ T cells (Davison, IAH) (Fig. 1.3). Numbers of $CD4^+$ cells increased slightly from 2 dpv, especially for fpIBD1Δ214::IL-18, compared to birds inoculated with PBSa and PHA. $CD4^+$ T cells gradually increased to reach a peak at 6 dpv at the site of vaccination in the skin, and then dropped in number gradually from 8 dpv onwards.

$CD8^+$ T cells. Skin sections were stained with the monoclonal antibody AV14, which recognizes $CD8^+$ T cells (5) (Fig. 1.4). $CD8^+$ cells increased rapidly and sharply starting from 4 dpv, reached a peak at 6 dpv at the site of vaccination in the skin, and then dropped rapidly from 8 dpv onwards.

B cells. Skin sections were stained with the monoclonal antibody AV20 which recognizes Bu-1 on B cells (6) (Fig. 1.5). B cells increased in number from 2 dpv, increased gradually to reach a peak at 6-8 dpv at the site of vaccination in the skin, and then dropped in number from 10 dpv onwards. At 2 and 4 dpv, B cells in skin sections were diffuse. The formation of germinal centres (GC)-like structures started at 6 dpv (Fig. 1.5). At 8 dpv onwards, almost all B cells were aggregated in these GC-like structures.

DISCUSSION

FPV has been used as a vector for over 20 years. However, to date surprisingly little data exist on the persistence of fowl pox vaccines in chicken tissues at the site of inoculation, or on what kind of local immune response there is to the vaccination at the site of vaccine inoculation. The results in this paper showed that the recombinant FPVs do not persist for long, and are cleared by 6 dpv.

It was showed that an intradermal inoculation of FPV (both challenge and vaccine strains) into the comb of birds resulted in primary lesions which were restricted to the site of inoculation (7). Local multiplication resulted in the development of lesions at the site of virus entry. Typically primary lesions appeared by the fourth day and disappeared by the third week. No secondary lesions developed in any of the birds. No virus was detected in the blood, and the internal organs were free of virus during the infection (7).

The challenge FPV was detected on the second day from the lesion samples collected from the comb (site of inoculation). Thereafter, the virus titer increased rapidly to a maximum on the ninth day, and then fell from the twelfth day onwards. No virus was detected on 27 dpi. Virus persistence was shorter and virus concentration was lower with the vaccine strain than the challenge strain (7).

Another study (8) showed that in chickens infected with fowl pox intradermally, the virus was detected in the lungs 4 dpi which was followed by viremia 5 dpi.

FPV strain FP9 was used as the recombinant vaccine in this study. Its genome has been completely sequenced (9), allowing identification of all of the differences (including deletions totalling 22 kb) between it and the USDA standard challenge virus, which was described in the original sequence publication as "pathogenic" (10). FP9 (FPV plaque 9) was derived by plaque purification of virus that had been passaged some 438 times in CEF culture, the source isolate being HP-1 Munich (11). Any residual virulence (even for day-old chicks) had been lost by passage 350 (12). FP9 is highly attenuated and possibly has introduced genetic deletions/modifications that enhance the immune response elicited by this vector. Comparison of the FP9 genome sequence with the published sequence of a pathogenic FPV reference strain (10) reveals several inserted as well as deleted sequences (9). Such deletions/insertions may account for the enhanced capacity of FP9 to elicit T cell responses. This may explain the short persistence of the recombinant FPV used in this study compared to the long persistence for the challenge FPV used by Minbay and Kreier (7).

In terms of histopathology, hyperplasia of the epithelium, enlargement of cells and the presence of eosinophilic cytoplasmic inclusion bodies (Bollinger bodies) are common for FPV infection (13). It was interesting to determine which cell types were involved at the site of FP9 inoculation.

By virtue of their location at various sites in the body, macrophages are one of the first cells to encounter viruses. The immunological function of the epidermis is principally linked to the presence in this tissue of a distinct subpopulation of Langerhans cells (LC) or macrophages. In man, LC constitute 2-4% of the epidermal cell population and within the epidermis they are the only cells which express MHC class II antigens constitutively (14). LC play a key role in the initiation of T cell responses to cutaneous antigens by picking up antigen and migrating to the draining lymph node (in mammals), where they trigger specific T cell activation.

In mammals, when an antigen has passed the epithelial barrier of the skin or mucosal surfaces it has to be processed and presented by accessory cells to lymphocytes. These reactions take place in lymphoid organs, such as the regional lymph nodes, Peyer's patches and tonsils, but also in the spleen if the antigen entered the blood directly. The respective lymphocyte clone expands by proliferating, and primed lymphocytes of the B and T cell series emigrate from the lymphoid organs.

As there are no lymph nodes in chickens, antigen presentation to lymphocytes may occur within the skin tissue. The virus was detected in skin tissue after vaccination at very high concentration at 2 dpv, to a lower degree at 4 dpv and was almost cleared from 6 dpv. The pattern of macrophage infiltration at the site of inoculation in the skin tissue was similar to that of the virus in that high numbers were seen at 2 dpv and 4 dpv, and these dropped from 6 dpv onwards. The concentration of macrophages in the skin at the site of inoculation for the six different viral vaccines is higher than that for the birds which were inoculated with PBSa (negative control) and PHA (inflammatory agent), suggesting that the virus might be taken up by macrophages. *In vitro*, PHA stimulates T cell proliferation and differentiation, while *in vivo* the PHA-skin response is an inflammatory reaction, concerning complex interactions of cells - not only lymphocytes but also macrophages and basophils (15).

The use of specific monoclonal antibodies, coupled with image analysis (Image pro-plus software) made it easy to assess changes in the quantity of target cells at the site of vaccination. The results of the experiments in this paper suggested that both $CD4^+$ and $CD8^+$ T cells participated in the response.

Intracellular organisms (usually viruses) will synthesize proteins inside the infected cells, and these

antigens therefore gain ready access to the MHC class I pathway; as a result, they often induce strong MHC class I restricted $CD8^+$ T cell responses (these cells are usually cytotoxic, although they also secrete a variety of cytokines in response to antigen).

There was a rapid increase of $CD8^+$ T cells in skin sections from 4 dpv, which reached a peak at 6 dpv, at which the virus was almost cleared. Furthermore, the antigens synthesized during intracellular infections usually will be released from the infected cells into the extracellular matrix, and these soluble materials are taken up by specialized antigen presenting cells (APC). Inside these APC, the antigens enter the MHC class II pathway, permitting them to induce MHC class II restricted $CD4^+$ T cells, which usually provide "help" to B cells. The soluble antigens will also encounter B lymphocytes, and therefore should (in concert with $CD4^+$ T cell "help") induce antibody responses. Therefore, we would expect most intracellular organisms (e.g. six different viral vaccines) to induce strong $CD4^+$ and $CD8^+$ cell responses, and B cell responses.

Recombinant FPV expressing tumour (16,17,18,19) and HIV antigens (20,21) elicit $CD8^+$ T cell responses in rodents. In addition, studies with nonhuman primates have shown that recombinant FPV encoding HIV antigens can boost the immune response primed by a DNA vaccine, leading to enhanced cytotoxic T cell responses and protection against viral challenge (20,22). Recombinant FPV vectors can be used to elicit $CD8^+$ T cell responses against *Plasmodium berghei* Malaria by prime boost immunization regimens using a novel attenuated FPV (23). Interestingly, FP9 was more effective in eliciting a response against the *Plasmodium berghei* circumsporozoite protein than the commercially available FPV vaccine strain.

B cells were not present in normal human skin (24). After vaccination, there was an aggregation of B cells at the site of vaccination. After antigen challenge, GC are formed within the secondary lymphoid organs (lymph nodes, Peyer's patches, spleen or tonsils) and in this experiment GC-like structures were formed in the skin tissue too (Fig. 1.5). At 2 and 4 dpv, B cells in skin sections were diffuse. However, the formation of GC-like structures started at 6 dpv and at 8 dpv onwards, almost all B cells were aggregated in these GC-like structures.

GC are the site where B cells grow and differentiate to immunoglobulin-producing plasma cells, generate high affinity antigen-specific B-cell receptors by affinity maturation and differentiate into memory cells. However, after fpIBD1 vaccination, detectible antibodies were produced against FPV but not against IBDV (2,25). One possibility is that fpIBD1 may not express correctly-folded VP2, due to the absence of VP4, or express it at very low levels so as not to stimulate an antibody response. The other possibility is that VP2 does stimulate cell-mediated immune responses which is crucial for protection against IBDV infection following vaccination with fpIBD1.

For successful vaccination, "pock" lesions should appear at the site of inoculation within the first week after vaccination. Immunity will normally develop at 10-14 dpv.

It was suggested that CMI plays a more important role in the recovery from FPV infection than humoral immunity (26). On the other hand, humoral immunity was not excluded since mortality was significantly higher in bursectomised-thymectomised chickens than in thymectomised chickens following FPV infection (26).

The regulation of the entry of activated T and B lymphocytes into skin tissue is not well understood. There is also evidence that keratinocytes participate in immune responses in the skin since these cells produce a wide variety of cytokines that can modulate T cell responses (27).

It would be very interesting to look for viral persistence and infiltration of immune cells at the site of inoculation after booster vaccination, as all the results in this paper reflected the response at the site of inoculation after a prime vaccination. It would also have been interesting to look for IL-18 expression in the skin, at the site of inoculation, after vaccination with different viral vaccines, to see if there were differences between viruses containing IL-18 and those without IL-18, and also to prove that IL-18 was expressed.

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Table 1. Primers and probes for real-time quantitative PCR.

RNA target	Primer/Probe*	Sequence (5'-3')
IBDV (VP2)	F	GAG GTG GCC GAC CTC AAC T
	R	AGC CCG GAT TAT GTC TTT GAA G
	Probe	TCC CCT GAA GAT TGC AGG AGC ATT TG

*The probe was labelled with FAM (5') and TAMRA (3').

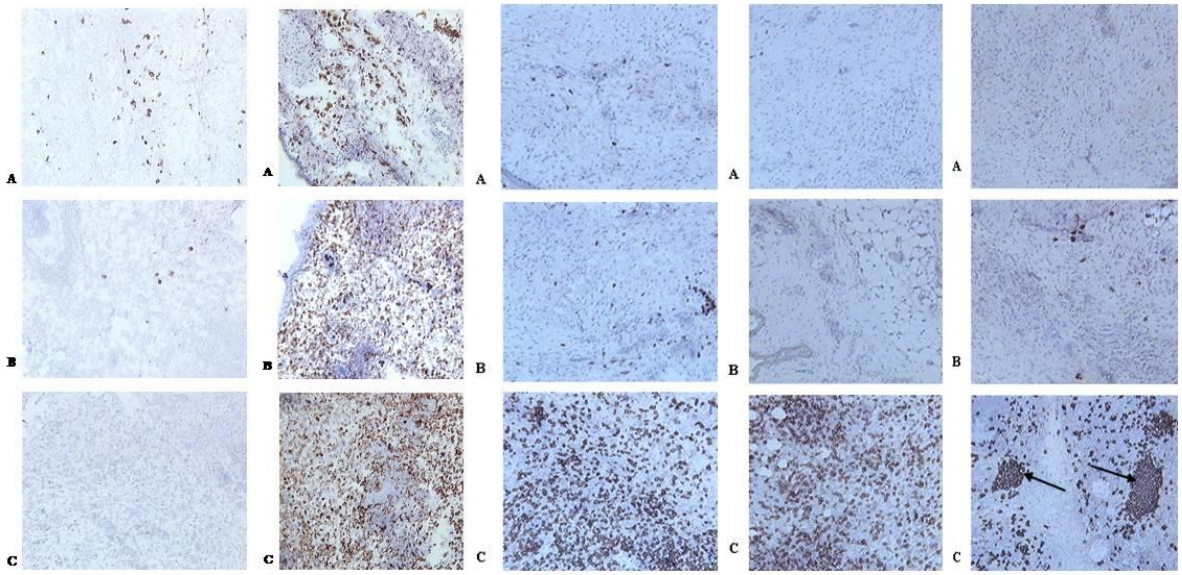

Fig.1 (1) Skin sections of birds vaccinated with fpIBD1 and stained with monoclonal antibody DF6 against FPV. (A) 2 dpv, (B) 4 dpv and (C) 6 dpv. The positive stained cells (brown) are the cells infected with the FPV (100x magnification).

Fig.1 (2) Skin sections of birds stained with monoclonal antibody KUL01 against macrophages. Two days post inoculation with (A) PBSa, (B) PHA and (C) FP9. Positive staining for macrophages is seen as brown (100x magnification).

Fig.1 (3) Skin sections of birds stained with the monoclonal antibody AV29, which recognizes $CD4^+$ cells, six days post inoculation with (A) PBSa, (B) PHA and (C) FP9. Positive staining for CD4+ cells is seen as brown (100x magnification).

Fig.1 (4) Skin sections of birds stained with the monoclonal antibody AV14, which recognizes $CD8^+$ cells, six days post inoculation with (A) PBSa, (B) PHA and (C) FP9. Positive staining for CD8+ cells is seen as brown (100x magnification).

Fig.1 (5) Skin sections of birds stained with the monoclonal antibody AV20, which recognizes Bu-1 on B cells, six days post inoculation with (A) PBSa, (B) PHA and (C) FP9. Positive staining for B cells is seen as brown. Arrows indicate formation of GC-like structures (100x magnification).

UNUSUAL vvIBDV OUTBREAK IN A FLOCK OF BROWN LEGHORN CHICKENS

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Infectious bursal disease virus (IBDV) or Gumboro disease virus is a viral pathogen of young chickens targeting differentiating B lymphocytes in the bursa of Fabricius. The virus is a non-enveloped, double-stranded RNA virus belonging to the family *Birnaviridae*. Infection with classical virulent strains of IBDV results in high morbidity and low mortality. Since the report of the emergence of very virulent IBDV (vvIBDV) in the 80s in Europe, the virus has spread to many countries. These strains can cause high mortality in chickens.

Here we describe vvIBD virus infection in a flock of Brown Leghorn pullets with mild increased mortality. Sixty live and dead, 6-10 week-old Brown Leghorn chickens from a flock of 100,000 pullets were examined because of generalized weakness and increased mortality.

Gross examination of the chickens showed markedly swollen and edematous bursas with prominent mucosal folds. Most bursas contained soft yellow necrotic exudate. Bursal hemorrhage was not a common observation. In addition, there were multifocal to coalescing petechial and ecchymotic hemorrhages in the skeletal muscles often on thigh muscles of the affected chickens. Histologically there was severe necrotizing bursitis with interstitial edema and lymphoid depletion. Lymphoid cell loss was also observed in periarterial lymphoid sheaths of the spleens. The birds did not have IBDV antibody titer.

Presence of vvIBD virus was confirmed by polymerase chain reaction (PCR) and virus isolation. Sequence analysis showed the virus grouped most closely with the sequence of 2008 vvIBD viral strain detected in California, however significant differences were seen suggesting a reassortant of vvIBD. In addition, lack of bursal hemorrhage and low mortality (~%3) were distinguishing features of this reassortant. The origin of this vvIBDV has not been determined yet. However, epidemiological factors of the spread of the disease are being investigated.

PRODUCTION LOSSES ASSOCIATED WITH "VARIANT" IBD ON BROILER PRODUCTION IN SASKATCHEWAN CANADA

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ABSTRACT

The objective of this study was to determine the economic impact of infectious bursal disease (IBD) on broiler production in Saskatchewan, Canada. A total of 177 barns from 58 premises were included in the study. The average size of a farm was 44,911 ft^2 (4177 m^2, or 0.4 ha) (range = 8,000 to 160,000 ft^2); and of those premises, 81% had single story barns, 8.6% had two story barns, and the remaining 10.4% had a combination of single and double story barns. The average placement on a premise was 69,500 broilers (range = 13,260 to 217,770). The majority of the farms (52%) had concrete floors, 31% farms had soil (dirt) floors, and the remaining 17% of farms had a combination of concrete/wood/soil floors. The average space per broiler was 0.64 ft^2 (0.06 m^2) (range = 0.40 to 0.94 ft^2). Vaccinations or medications at the hatchery were as follows: antibiotic injections (29%), Marek's vaccination (10.3%), infectious bronchitis vaccination

(34.5%), and infectious bursal disease vaccination (6.9%). The average age at processing of a broiler was 37 d (range = 33 to 45 d). The average market weight of a broiler was 2.03 kg (range = 1.6 to 2.58 kg). The average daily gain of a broiler was 55 g/day (range = 43 to 64 g/day). The average feed conversion efficiency ratio (FCR) of a broiler was 1.83 (range = 1.59 to 2.22). The average broiler production (kg live market weight/m^2 floor area) was 32.36 kg (range = 21.48 to 49.60 kg). Sixty-four percent of premises had an interval of more than 10 d "downtime" (range = 0 to 21 d). IBD infection was demonstrated by RT-PCR at 36% of premises. Genotyped IBD viruses were Del E, 586 and NC171 like variants. The premises with no IBD infections had a lower FCR (1.81) than the premises with IBD infections (1.87). The premises with no IBD infection had a higher broiler production (kg/m^2) (33.35 kg) than the premises with IBD infections (30.63 kg). The livability or percentage of birds marketed at processing was higher in premises with no IBD infections (93.9%) than the premises with IBD infections (91.4%). In summary, FCR was decreased in farms with no IBD infections, concrete floors, more than nine days of downtime. The livability was highest in farms with no IBD infections. Broiler production (kg/m^2) was highest in farms with no IBD and in new barns. Based on the above results it was calculated that variant IBD reduced broiler production by at least 160,000 kg in Saskatchewan for the year of the study.

INTRODUCTION

Infectious bursal disease virus (IBDV) belongs to the family Birnaviridae, an acute highly contagious disease of two to six week-old chickens (1,2). The most severe consequence of IBDV infection is the functional loss of the bursa of Fabricius (BF). Subtypes of IBDV emerged that could not be controlled by immunization with vaccines prepared from ''classic'' IBDV strains, and were called "variants'' (4) These IBDVs escape the immunity elicited by classic vaccines (5,6). "Variant" strains do not cause obvious clinical disease, but induce severe immunosuppression. The immunosuppression resulting from an IBDV infection is the underlying cause of many cases of respiratory and enteric disease in chickens and vaccination failures. Vaccination with attenuated virus is widely used worldwide to control IBD in broilers (7). One of the major problems with attenuated IBD vaccines is their sensitivity to maternally derived antibodies which are always present at the time of vaccination. One way to approach this issue is the use of less attenuated "intermediate" vaccines, but these vaccines can themselves cause a degree of vaccine-induced lesions in the BF (8).Because the IBD virus is resistant to many disinfectants and environmental factors, once a poultry house becomes contaminated, the disease tends to recur in subsequent flocks. Control of IBD has been complicated by the recognition of "variant" strains of the IBD virus in broiler barns in many provinces of Canada (9). The objective of this study was to identify the economic impact of "variant" IBD in the Saskatchewan broiler industry.

MATERIALS AND METHODS

A total of 177 broiler flocks (barns) from 58 premises (farms) in Saskatchewan were included in this study. Data were collected from one production cycle (A19) during April-June, 2009. Of 58 farms, 10 farms had a single barn, 15 farms had two barns, 18 farms had three barns, 10 farms had four barns, and the remaining farms had five to13 barns. Thirty-six of 58 farms had 10 or more years old barns and 22 of 58 farms had one to nine year old barns. Forty-seven of 58 farms had single story barns, five of 58 farms had double-story barns and the remaining five farms had both single and double story barns. Twenty-seven of 58 farms had concrete floors and the remaining farms had combination of partial concrete, wood or soil floors. All broiler placements in Saskatchewan were supplied by two hatcheries, and all broiler flocks in Saskatchewan were processed by two processing plants. Mortality, feed conversion ratio (FCR), daily weight gain, broiler production (kg/m^2), vaccination, impact of IBD infection, and condemnation data were collected from all 58 farms.

Bursae of Fabricius were collected from 74 randomly selected barns (20 birds/ barn) in 26 of 58 farms at the time of processing. The bursal and body weight of individual birds were measured to calculate bursal weight to bursal weight ratio. Histopathology of bursae was performed to evaluate bursal atrophy associated with IBD. Blood samples (20 samples / barn) were taken from 52 of 74 randomly selected barns from 26 of 58 farms at the time of processing for IBDV titers (IDEXX, Westbrook, ME). Farms with bursal atrophy on gross and histopathological examination and low body weight to bursal weight ratio were further examined by collecting bursae from ten randomly selected birds at d 19 post-hatch for virus isolation and genotyping at the Animal Health Laboratory, University of Guelph.

RESULTS AND DISCUSSION

The average floor area of a farm was 4,147 m^2 and the range between 744 and 14,880 m^2. The average placement in a farm was 69,048 birds and the range between 13,000 and 217,770 birds. The average bird density was 0.64 ft^2/bird (0.06 m^2/bird) and the range

between 0.40 and 0.94 ft^2 per bird. The average age at marketing was 37 d and the range between 33 and 45 d. The average market weight of a bird was 2.03 kg and the range between 1.60 and 2.58 kg. The daily weight gain was 54.4 g/day and the range between 43.48 and 64.40 g/day. The average FCR was 1.83 and the range was 1.59 to 2.22. The average live bird production was 32.36 kg/m^2 and the range between 21.48 and 49.60 kg/m^2. Thirty-six of 58 farms maintained nine days or more down time between flocks following completion of cleaning and disinfection. Twenty of 58 premises practiced vaccination against infectious bronchitis virus (IBV) (MildVac-M, Intervet Canada Ltd., ON), six of 58 premises practiced vaccination against Marek's disease (MD), and four of 58 premises practiced vaccination against IBD. Seventeen of 58 farms used *in ovo* injected antibiotics.

IBD was present in 21 of 58 (36%) farms based on histopathology of bursa of Fabricius, bursal weight to body weight ratio, and antibody titers against IBD. Variant IBDV NC171, 286 and Del E were isolated from the bursae of day-19-old birds. The average production was 33.35 kg/m^2 in farms with no IBD and the production was 30.63 kg/m^2 in farms with "variant" IBD. FCR was 1.87 in farms with IBD and FCR was 1.81 in farms with no IBD. The average mortality (including culls) was 8.6% in farms with IBD infection in contrast the average mortality was 6.1% in farms with no IBD infection. The bursal weight to body weight ratio was 0.17% (range 0.21 to 0.12%) in farms with no IBD in contrast bursal weight to body weight ratio was 0.06% (0.12 to 0.04%) in farms with IBD. The mean (geometric mean) IBD titer in barns with no IBD was 302 (range 1939 to 2) in contrast barns with IBD geometric mean was 3835 (range 7466 to 179). The bursal histology score [bursal atrophy range (0 to 60)] was 3.1 (range 20.3 to 0.5) in farms with no IBD in contrast bursal histology score was 51.9 (range 58.8 to 36) in farms with IBD challenge. IBD infection was common in old barns [17 of 36 (47%)] compared to new barns [four of 22 (18%)].

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USING COMPUTER IMAGING ANALYSIS TO PREDICT FLOCKS THAT WILL BE PCR POSITIVE FOR IBDV

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INTRODUCTION

Infectious bursal disease virus (IBDV) surveys are commonplace in the poultry industry. Typically, several broiler flocks from a given production complex are analyzed by fixing bursas in formalin. After H&E staining of the fixed and sectioned bursa tissues, they are examined microscopically and either scored by a pathologist or by using computer imaging analysis. Computer imaging takes advantage of the H&E staining contrast between B-lymphocytes (dark blue) and the underlying architecture (pink) of the bursa of Fabricius. Thus, the computer will score a darker staining "intact" bursa higher than one showing significant lymphoid depletion. While the primary use of this technology is to estimate the timing of IBDV field infections, it also can be used to determine which flocks are good candidates for further testing using PCR analysis and/or virus isolation. If calibrated properly, computer imaging analysis can cut down on

the testing of negative samples, which in turn can lead to significant cost savings.

MATERIALS AND METHODS

Five bursas were collected and pooled from twenty healthy, three-week-old broiler flocks at 15 different complexes (300 total flocks). All bursas were split lengthwise and their respective halves were 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). A flock was suspected to be positive for IBDV if it contained at least one bursa with an imaging score of 26% or lower (significant depletion) or at least two bursas with an imaging score of 35% or lower (not fully intact). To test this cut-off criteria, several "intact" flocks were also submitted for PCR testing.

RESULTS

Three complexes were not submitted for PCR testing – one because paired frozen tissues were lost and two because bursa from all flocks were intact based on computer imaging. Of the remaining 12 complexes (240 flocks), a total of 91 flocks were tested for IBDV by PCR analysis. Thirty-three flocks tested positive and 58 tested negative for IBDV. Based on our computer imaging cut-off criteria, 36 flocks were considered suspect positive prior to PCR testing. Of those, 31 were in fact PCR positive while five were PCR negative, for a rate of 86% correctly predicted to be positive (see Table 1). In contrast, of the 55 submitted that were suspected to be IBDV negative based on computer imaging analysis, 53 were indeed negative while two were PCR positive (4%).

DISCUSSION

This large bursal field survey demonstrates that lymphocytic depletion measured by computer imaging analysis can do a reasonable job of predicting which flock samples will test either positive or negative for IBDV using PCR analysis. This allows one to focus on the samples that are most likely to yield a positive PCR test result. In this particular survey, 239/300 (80%) of the flocks were deemed completely intact based on computer imaging. Of these 239 intact flocks, 49 were tested and all were confirmed PCR negative (see table). Of the 36 flocks that were suspected positive for IBDV, 86% of those flocks yielded a positive PCR result. Put another way, only 14% of the expected positive samples yielded a negative PCR result, compared to over an 80% negative yield if all samples in the survey had been indiscriminately tested.

As is often the case, the exceptions or incorrectly predicted flocks best illustrate the potential limitations or nuances of the assays. There were four PCR-tested flocks that had just one bursa with mild depletion (score 30-35%). Of these four, one turned out being IBDV PCR positive. We don't usually PCR test these "borderline" imaging profile flocks because in the early stages of an IBDV infection, the relatively small amount of IBDV in the one bursa can often be diluted by the other intact bursas. In contrast, samples with at least two mildly depleted bursas or one significantly depleted bursa usually contain enough IBDV to yield a positive PCR result.

So, then what can we make of the five false negative results in the table?

1) Although not often seen in the United States, other immunosuppressive viruses such as Marek's disease virus and reoviruses can cause significant lymphoid depletion in broilers (3);

2) improper handling of samples (elevated temperature and/or bacterial overgrowth) at any stage between the collection of the tissues to the prepping of the samples for PCR testing can compromise the integrity of the viral RNA; and

3) although the IBDV PCR primers bind to an extremely conserved region of VP2, there is always a remote possibility that a field virus has acquired a novel mutation (4) that would prevent the primers from annealing and yielding a PCR product.

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Table 1. Rate at which computer imaging analysis correctly predicted the outcome of PCR test results.

Flock categories based on Computer Imaging Analysis		Flock test results		Total #tests	Prediction rate
Group category	Defining criteria	PCR negative	PCR positive		
Suspect IBDV+	>1 bursa score <27%, or 2 scores <36%	5	31	36	86%
Suspect IBDV-	No more than 1 bursa w/ score 30-35%	53	2	55	96%
Completely intact	All scores >35%	49	0	48	100%

TRANSCRITPOME SEQUENCING ANALYSIS OF AN INFECTED BURSA OF FABRICIUS

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The virus infects young chickens and brings immunosuppression and mortality at three to six weeks of age. Previously, with the use of cDNA microarray, we demonstrated that a number of transcripts expressed differentially in IBDV-infected chicken embryonic fibroblasts. In general, cell surface receptors involved in B cell and T cell activation and differentiation were suppressed, while the target genes and inducers of NF-kB and the genes involved in toll-like receptor- and interferon-mediated responses were up-regulated. Though cDNA microassay assay provides a general picture on *in vitro* host cell responses in IBDV infection, there are still many information gaps in the pathogenesis pathway to be completed. We conducted deep transcriptome sequencing on the IBDV infected bursa of Fabricius. Entire bursas were collected from both healthy and infected chickens that showed typical Gumboro symptoms at 20 d of age. Total RNA and poly-A+ RNA were isolated from the organ followed by double-stranded cDNA synthesis. Complementary DNA libraries were then constructed followed by emulsion PCR amplification. Sequences of these transcriptomes were then analyzed by 454 GS Junior System. After sequence assembly, more than 3000 contigs were obtained in each transciptome and the sequences of contigs were blasted against public databases. Apart from the identification of transcript species, expression level of each transcript was also reflected by the magnitude of coverage in each sequence contig. To conclude, we made use of deep transcritpome sequencing method to identify the difference between the entire transcritpome of control and infected chicken bursa, and hence provide detail information on the host cell defense and viral pathogenic mechanisms upon IBDV infection.

(The full manuscript will be submitted for publication to the *Journal of General Virology*.)

INFECTIOUS BURSAL DISEASE: EVALUATION OF THE PATHOGENICITY AND IMMUNOGENICITY OF COMMERCIAL VACCINES IN BRAZIL

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SUMMARY

This study characterized the pathogenicity and immunogenicity of three vaccines against infectious bursal disease (IBD): Recombinant vaccine, immune-complex and intermediate. Four groups, with sixty specific pathogen free (SPF) chickens each, were vaccinated on the first day and challenged on the 25th. Chickens were examined and necropsied on the 25th, 30rd and 35th d. The bursae of Fabricius (BF) were collected, weighed. Their diameters and relative weights were measured and subjected to histological examination by digital analysis. Serum samples were subjected to ELISA for quantification of antibodies against IBD. At 23 d of age, the birds were submitted to the cutaneous basophil hypersensitivity (CBH) test to evaluate cell-mediated immunity. The results showed that the immune-complex vaccine induced, in the BF, the largest reduction in relative weight, diameter and greater microscopic lesion score. The recombinant vaccine showed better results in the evaluations of BF. In the serological analysis, before the challenge, intermediate vaccine induced a greater production of antibodies compared to the recombinant vaccine. The recombinant vaccines and intermediate remained differing among themselves only up to five d post-challenge, because, after 10 days of challenge, all three vaccines showed similar antibody titers. The evaluation of cell-mediated immunity showed no difference among the vaccines, but the cell-mediated immune reaction of the recombinant and immune-complex vaccines was smaller than the non-vaccinated group. However, immune-complex and intermediate vaccines caused the highest lesion degrees in the BF. The recombinant vaccine didn't induce lesions in the BF. All vaccines were effective in protecting chickens against the challenge with IBDV.

INTRODUCTION

Infectious bursal disease virus (IBDV) is an important immunosuppressive virus of chickens. The immunosuppressive effects are most pronounced when exposure to the virus occurs in the first two to three weeks of age (1), during which the bursa of Fabricius (BF) reaches its maximum cell development (7). Infectious bursal disease virus infection, depending on the virulence of the strain, may exacerbate infections with other infectious agents and reduce the ability of the immune system to respond to vaccinations (9), since the virus compromises the humoral and cellular immune responses of chickens. Inhibition of humoral immunity is attributed to the destruction of IgM + B lymphocytes from BF by lytic infection caused by viruses (4). There is strong evidence that IBDV infection also causes severe impairment of in vitro mitogenic proliferation of T cells in the affected birds, returning to normal levels after five or more days of infection (9).

The IBDV is highly contagious and resistant. Therefore, despite the adoption of strict hygiene measures, vaccination is the main way to control the infection of chickens during first weeks of life. However, the emergence of highly virulent strains of IBDV, which break the barrier of vaccination, the knowledge that "strong" live vaccines induce damages to the BF in the same intensity as wild strains of IBDV and the interference of maternal antibodies on vaccination of young chickens have induced animal health laboratories to develop new vaccines with different vaccination strategies in an attempt to circumvent these obstacles. Among them, recombinant and immune-complex vaccines can be highlighted. The recombinant vaccine uses a vector virus to hold and express the immunogenic VP2 protein of IBDV, inducing, even with the presence of maternal antibodies, the immune system to produce specific immune cells against the IBD. Yet, the immune-complex vaccine has as innovation the fact that the vaccine virus is coated with anti-IBDV antibodies, temporarily protecting the vaccine virus against the neutralization by maternal antibodies.

The objective of this study was to characterize the pathogenicity and immunogenicity of three commercial vaccines against IBD in specific-pathogen-free (SPF) chickens, when used on the first day, which are: a herpes virus of turkey (HVT) recombinant vaccine expressing the immunogenic protein 2 of a classic

IBDV strain (Faragher 52/70), a immune-complex vaccine (Winterfield 2512 strain) and an intermediate vaccine (Lukert strain).

MATERIALS AND METHODS

The experimental groups with 60 SPF White Leghorn chickens each consisted of:

G1: non-vaccinated chickens (negative control);

G2: chickens immunized with the recombinant vaccine (HVT + VP2);

G3: chickens immunized with the immune-complex vaccine (Winterfield 2512); and

G4: chickens immunized with an intermediate vaccine (Lukert strain).

The birds in each group were randomly distributed to cages with five floors each (total of 12 chickens per floor), placed in individual isolation units where water and feed were provided *ad libitum*.

The SPF chickens were vaccinated during their first days of life and, after 25 d, challenged with a very virulent strain of IBDV, corresponding to the genomic group 11 (G11) (3), with 100µL of inoculum per chicken prepared to 10^4 embryo infectious dose (DIE_{50})/mL, ocular route.

At 25, 30, and 35 d old, 20 birds in each group were examined, weighed, bled by cardiac puncture, euthanized by electrocution, and necropsied. Bursas of Fabricius were collected, weighed, their diameters measured, evaluated regarding the presence of macroscopic lesions, fixed in 10% buffered formalin, and subjected to histological examination by a digital analysis of lymphoid depletion (ADDL, in Portuguese), as proposed by Moraes *et al*. (6).

The bursa/body weight (B/B) ratio was determined by the following formula: bursa weight (in g)/body weight (in g) x 1000. BF diameter was measured using a caliper with 0.01 mm accuracy. Blood samples were sent for ELISA indirect serological analysis with a commercial kit (IDEXX® FlockChek IBD).

To assess cell-mediated immune response, SPF immunized and non-immunized chickens were submitted to proof of cutaneous basophil hypersensitivity (CBH), consisting of intradermal injection (in the interdigital space between the 3rd and 4th toe on the right paw) with 100µL of phytohemagglutinin-P (PHA-P) and the measurement of reaction (edema), with the help of a digital caliper, at 0 h, 12 h, and 24 h later. The same procedures were performed in the left paw with PBS (phosphate buffered saline). The formula for calculating the cellular immune response was: (1) response to PHA-P = thickness post-injection of PHA-P on right paw - thickness pre-injection of PHA-P on right paw; (2) control response PBS = thickness post-injection of PBS on left paw - thickness pre-injection of PBS on left paw. Thus, the cellular reaction at all times was obtained by: CBH = 1 - 2 (10).

The following computer programs were used for statistical analysis of results: JMP™, version 6.0 and SAS Institute inc. (2005)

RESULTS

In immunized SPF chickens, no clinical signs or mortality were noted before or after the challenge. However birds of group 1, non-vaccinated, had severe clinical signs (100%) and high mortality (66%) after challenge.

Table 1 shows data on diameter, relative weight, histological examination and serological analysis of BF, according to the analysis period and the vaccine used for the immunization of SPF chickens.

Cell-mediated immunity. The measure of reaction to PHA-P 12h post-injection showed that the birds of G2 had statistically ($P \leq 0.05$) a lower cellular immune reaction (0.1684 mm) when compared to the birds from G1 (0.3033mm); however, they were not statistically different ($P \leq 0.05$) from birds in other groups (G3 and G4). In the evaluation at 24 h post-injection, groups 2 and 3 had statistically ($P \leq 0.05$) a lower cellular reaction (0.1584 and 0.1771mm, respectively) when compared to G1, which showed a reaction of 0,2835mm. The vaccination groups (G2 to G4) did not differ ($P \leq 0.05$) among themselves and G4 (0.2089mm) did not differ from G1 as well.

DISCUSSION

Analyzing data from the evaluation of diameter and relative weight of BF, we found that the group of chickens immunized with the immune-complex vaccine had the lowest values, which is explained by the fact that the vaccine consists of a strong strain (Winterfield 2512) of IBDV, because the BF suffered atrophy, even before the challenge with the vaccine virus infection. Furthermore, the results of the ADDLs support these findings, since the chickens immunized with the immune-complex vaccine showed higher lesion scores in BF, after challenge, in comparison with other vaccination groups (intermediate and recombinant vaccines). They have also shown lesion scores similar to the negative control group (G1) at 10 d post-challenge (PD). Haddad *et al*. (2) also found lower values for the relative weight of BF in chickens immunized on the first day with the immune-complex vaccine, even in the presence of maternal antibodies. Similar data was also reported by Donald *et al*. (5), for SPF chickens vaccinated in ovo and challenged at 21 d old, showed BF relative weight lower than that of the non-vaccinated ones.

Unlike the immune-complex vaccine, the recombinant vaccine achieved the best results in relation to diameter, B/B ratio and lesion scores (ADDL). These findings are explained by the fact that, besides the vaccination protection against birds challenge, it consists of the VP2 subunit of IBDV and, therefore, it did not cause cellular damage to BFs. The results with the HVT + VP2 vaccine are similar to those reported by Perozo *et al*. (8), in which SPF chickens challenged with the variant E of IBDV supported the infection, showing low BF lesion scores and high values for B/B ratio. Similarly, Tsukamoto *et al*. (12), did not report any degree of BF lesion in SPF chickens, during a test with a recombinant HVT + VP2 vaccine (built with Pec promoter responsible for the VP2 gene transcription), challenged with E/91 strain of IBDV.

The intermediate vaccine showed results similar to those of the recombinant vaccine in relation to BF lesion scores, differing only in diameter and B/B ratio, which is due to the BF atrophy caused by the vaccine virus infection before challenge.

The serological analysis showed that the recombinant vaccine induced a lower production of antibodies against IBD when compared with intermediate vaccine; however, it was sufficient to protect the SPF chickens from the challenge, as evidenced through the evaluations of BFs. Tsukamoto *et al*. (11) also found lower antibody titers in birds immunized with a recombinant vaccine against IBD (rMDV - Marek's disease virus serotype 1 expressing VP2) in relation to the birds immunized with an intermediate vaccine. However, the rMDV vaccine tested protected only 55% of the birds against the challenge with a very virulent IBDV strain. Intermediate and immune-complex vaccines also induced the production of antibodies sufficient to protect birds from challenge, because damages to BFs were caused by the vaccine virus infection prior to the challenge.

CONCLUSIONS

This experiment showed that the three vaccines tested induced the immune system of SPF chickens to produce defense cells against the IBD sufficient to protect them from the challenge and that the damage to the bursa of Fabricius increases according to the virulence of the strain used in preparing the vaccine. The recombinant vaccine may be the best alternative, since it does not induce to lesions in the bursa of Fabricius.

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Table 1. Diameter, relative weight, BF lesion scores and serological evaluation in periods of 25, 30 and 35 d old of SPF chickens vaccinated on the first day and non-vaccinated.

Period	Group	Vaccination	D* (mm)	B/B ratio (%)	Bursal lesion scores	ELISA (Log 10)
25 d PV	1	NV	12.77^{A}	$6.05^{A,B}$	1.00^{B}	0.59^{C}
	2	RC	12.91^{A}	7.17^{A}	1.26^{AB}	2.35^{B}
	3	IC	07.83^{B}	2.13^{C}	1.93^{A}	2.92^{AB}
	4	IN	11.18^{A}	5.05^{B}	1.33^{AB}	2.99^{A}
5 d PD	1	NV	12.84^{B}	6.23^{AB}	3.36^{A}	-
	2	RC	15.28^{A}	7.32^{A}	1.38^{C}	2.84^{B}
	3	IC	10.26^{C}	2.67^{C}	2.15^{B}	3.16^{A}
	4	IN	13.86^{AB}	5.87^{B}	1.09^{C}	3.38^{A}
10 d PD	1	NV	08.38^{C}	1.54^{C}	3.36^{A}	3.45^{A}
	2	RC	16.81^{A}	7.72^{A}	1.31^{C}	3.01^{B}
	3	IC	09.95^{C}	2.18^{C}	1.93^{B}	3.16^{AB}
	4	IN	14.64^{B}	6.25^{B}	1.04^{C}	3.29^{AB}

Vaccines: NV - non-vaccinated; RC - Recombinant; IC - immune-complex; and IN - Intermediate.
D# = BF diameter; B/B ratio = Bursa weight:body weight X 1000;
A, B or C Means with the same letter within the column are not significantly different ($P \leq 0.05$).

INFECTIOUS BURSAL DISEASE AND ITS CONTROL IN THE FIJI ISLANDS

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SUMMARY

New Zealand is free of IBD and vaccination against the disease is not permitted. This results in the export of day old chicks and hatching eggs lacking IBD maternal antibodies to Pacific Islands, notably Fiji. This has produced challenges in controlling IBD, but also an insight into IBD pathogenesis under unique field conditions. This paper discusses the control options tried, their varying degrees of success, and invites suggestions as to where we might go next.

Fiji is an island nation in the Pacific Ocean, 2000 miles north of New Zealand (NZ). By world standards Fiji's poultry industry is tiny, but with only 900,000 people and broiler production of 250,000 birds a week, and about 300,000 commercial egg layers, poultry makes up over 80% of livestock production by value. Traditionally the Fiji broiler industry sourced day old chicks from local, Australian and NZ eggs. In the early 90s, a cyclone destroyed the local breeder farm, and it was decided that 50% of hatching eggs would in future be sourced from overseas. Avian influenza and Newcastle disease outbreaks in Australia closed off that source, resulting in large numbers of New Zealand fertile eggs being imported. NZ is free of IBD (1), and vaccination against the disease is not permitted. Whilst IBD freedom allows the New Zealand broiler industry to achieve world-beating performance, it results in the export of day old chicks and hatching eggs lacking maternal antibodies to IBD to Pacific Islands, notably Fiji, where IBD is endemic. This has produced added challenges in controlling IBD and diseases secondary

to immune suppression, but it gives an insight into IBD pathogenesis under unique field conditions.

In 1993 New Zealand had suffered an outbreak of IBD, apparently related to contamination of a batch of Marek's vaccine with IBD vaccinal virus, and as part of the (successful) eradication campaign, inactivated IBD vaccines were used in NZ breeder flocks in 1998-2000. During this time many of the eggs sent to Fiji carried maternal antibodies against IBD, but after late 2000, all eggs were free of maternal antibodies to IBD.

A new breeder farm was established in Fiji in 1998, and from mid-2001, it became apparent that the mortality rate in birds from local eggs from IBD vaccinated-parents was less than half that of birds from New Zealand eggs.

Infectious bursal disease. Maternal antibody free, NZ-source broilers show no clinical signs of infection with IBD virus; however, by seven days of age their bursas are half the size of Fiji-source chicks' bursas; and histologically, bursal damage is detectable as early as three days post placement. In Fiji-source birds, it is occasionally possible to detect slight bursal edema at about 14 days in birds culled for leg deformities, indicating that maternal antibody protection lasts until 11-12 days. IBD virus was confirmed by immunoperoxidase staining in selected bursal slides using murine monoclonal antibody CSIRO/IBDV/17-82 (Tropbio Pty Ltd, Australia), but correlation between presence of detectable IBD virus and a score of histological bursal damage (2) was unreliable.

Serological testing using IDEXX ELISA kits shows that IBD maternal antibodies in local chicks decline to a minimum at 20 days of age. A serological response in NZ-source birds is not detected before 15 days of age, from where antibody levels climb to higher titers than in local chicks.

Inclusion body hepatitis. IBH is the main cause of death in Fijian broiler flocks. Deaths due to IBH begin from 15 days in NZ source chicks and continue until day 26 to 30. *E coli* septicemia contributes to further elevation of death rates in a minority of flocks after day 25. Clinical presentation is as described (3) for South American outbreaks; morbidity is not high but affected birds are obviously febrile. At post mortem examination they typically have friable, gun-metal color livers and jaundice, accompanied by enlarged spleens, small thymuses, pale kidneys, and small bursas. Hydropericardium has not been a feature of IBH outbreaks in Fiji.

Histologically inclusion bodies are basophilic and can infect up to 25% of hepatocytes.

The bursas of birds dying from IBH typically have fewer than 20% functional follicles. Birds culled for other reasons had more than 20% functional bursal follicles.

Subclinical effects on flock growth rates add about one day to growout period, an effect postulated (4) to be due to impaired hepatitc function. Liver condemnation rates have gone up as the problem has increased in local source birds. The Fiji adenovirus been shown to preciptitate liver rupture and hemorrhage at 25 days in fast-growing birds. Histological examination of affected livers (which showed no obvious signs of IBH) revealed that up to 15% of hepatocytes contained basophilic adenoviral intra-nuclear inclusion bodies. The infection constrains the ability of hepatocytes to store and utilize triglycerides, functions required to obtain optimum performance from high-energy diets.

CONTROL EFFORTS

IBD vaccination of broilers. All permutations of vaccination have been tried in the field to protect NZ-source broilers. PBG 98 vaccine (very mild) was used at day old; Vibursa-L (mild, Lohman), in the drinking water at day old, full dose, half dose, or by subcutaneous injection; Bursine 2 (Fort Dodge) full dose, half dose at day old and at eight days.

Numerous trials confirm that day-old vaccination with conventional vaccines offers no protection, which is to be expected as there is no time for the vaccine to multiply in the birds prior to them receiving a field challenge of thousands of times more virus particles within an hour of the vaccination being administered (all the broiler houses are within an hour's drive from the hatchery).

In ovo vaccination is not considered a viable option with the current hatchery set-up; two setter tray sizes, and a throughput of only 250,000 birds per week makes this uneconomical, before getting equipment maintained and serviced in a remote Pacific Island are considered. What has been tried is spray vaccinating birds with Vi-bursa-L at about 12 h prior to the hatch being pulled in order to give the vaccine virus a "head start." The time the procedure took affected the hatcher temperature, and a lack of clear benefits resulted in the trials being abandoned.

When imported eggs could be set on a Monday, chicks were vaccinated on take-off and holding them in a secure room overnight prior to placement. Some benefits were seen, but when more imported eggs were used than could be set on one day, this became logistically difficult too.

Hygiene and cleanout constraints. Improvements in hygiene at placement work. This has been shown by the fact that imports of day old parent chicks from New Zealand that are placed in spelled, disinfected, and fumigated houses do not suffer elevated mortality or show the same level of bursal destruction. Day old commercial layers imported as chicks from New

Zealand and placed in well-sanitized brooders also escape significant effects from IBD. The broiler farms have mainly dirt floors, and though the litter is removed, they are impossible to clean, and shed turnaround times are very short. A*lphitobius* beetles, which have been shown to harbor IBD virus, are a problem.

Injection of day old chicks with hyperimmune serum collected at slaughter. Trials with this were carried out on a number of flocks (about 140,000 birds) with favorable results, but the process is unforgiving if the sterility of the serum is not maintained.

Maternal antibody protection of local-source chicks. The industry has followed the path of increasing the proportion of locally-produced broilers from hens that have received a comprehensive IBD vaccination program.

D/o Vibursa L

two weeks, five weeks Bursine 2

12 weeks, 18 weeks Bursine K.

This worked well in Fiji-source chicks until about 18 months ago, when death rates from IBH in NZ-source chicks rose to higher levels (20% in some flocks), and local chicks started to show an increase in mortality from IBH. This poses questions as to whether we are dealing with a hotter strain of IBD virus, or whether the IBH virus is pathogenic in its own right, or has the standard of vaccination fallen in local breeders.

Where to from here? The first step is to have IBD virus re-typed and modify vaccination programs if required. Characterizing the adenovirus will also be useful. The vaccination procedures for parent flocks in Fiji are being tightened to ensure that maternal antibody levels are maximized.

Given the obvious deficit in maternal antibodies in NZ-source chicks, there have been discussions around using virus-antibody complex vaccines by injection at day old in both local and NZ-source day old chicks. Advice received has been to avoid these as we would be introducing Intermediate plus IBD vaccine to the islands. Attempts to obtain a supply of the freeze dried serum have been unsuccessful. Do we make another attempt to make our own hyperimune serum?

Not previously considered is the use of an HVT vectored IBD vaccine, which would have to be administered at day old. The birds are slaughtered at young ages (35-40 d).

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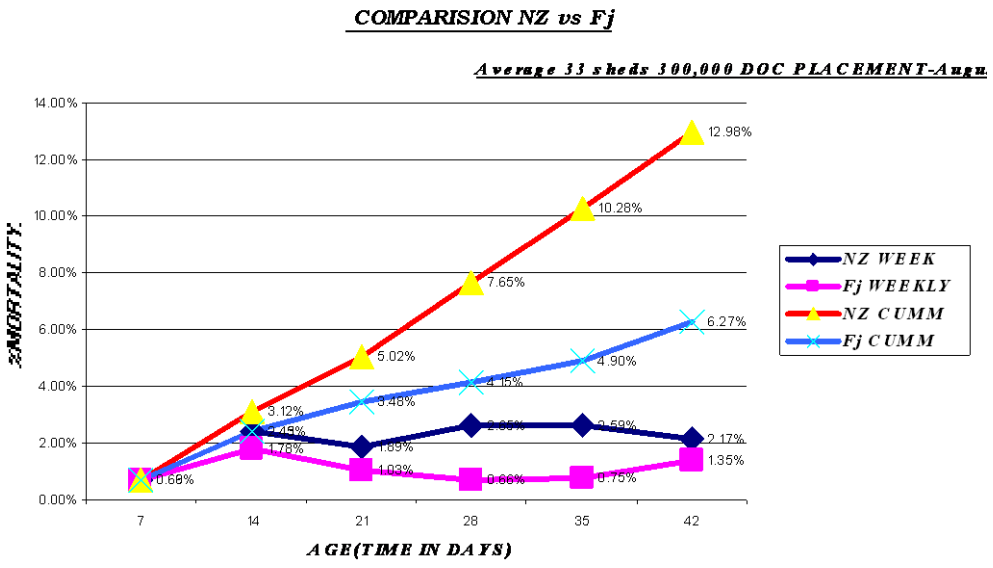

DETECTION OF VARIANT STRAINS OF INFECTIOUS BURSAL DISEASE VIRUS IN BROILER FLOCKS IN SAUDI ARABIA USING ANTIGEN CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

Infectious bursal disease (IBD) conditions were observed in 15 commercial and nine backyard broiler flocks in central area of Saudi Arabia during 2007-2008. The age of birds ranged from two to eight weeks. The size of commercial flocks ranged from 5000 to 15000 birds and these flocks were vaccinated with classical strain of IBD vaccine at 14 days of age through drinking water. Number of birds in backyard flocks ranged from 200 to 300 and the vaccination program of these birds was not known. High mortalities, respiratory symptoms, stunting and enlargement of bursa were seen in diseased birds of commercial flocks. Infectious bursal disease was suspected based on these clinical symptoms and postmortem findings, although these birds had been vaccinated against IBD virus. In order to confirm our diagnosis and to identify the causative agent, antigen capture-enzyme linked immunosorbent assay (AC-ELISA) was carried out on 142 bursal samples collected from diseased birds using kits containing monoclonal antibodies against variant strains of IBDV and 61.23% samples were found positive. It was observed that traditional vaccinal strains (54.02%) were significantly higher than less pathogenic strains not used in vaccine preparation (29.89%) and nontraditional highly pathogenic strains of IBDV (16.09%). It was concluded that new variant strains of IBDV were detected in the samples in Saudi Arabia and to our knowledge this is the first report about the existence of these virus strains in commercial and backyard broiler flocks in this country.

INTRODUCTION

Infectious bursal disease (IBD) has been of great concern to the poultry industry for a long time, particularly in the past decade. Indeed, its re-emergence in variant or highly virulent forms has resulted in significant economic losses (6). The etiological agent of IBD, infectious bursal disease virus (IBDV), is a non-enveloped virus, belonging to the family Birnaviridae, with a isegmented dsRNA genome (6). In fully susceptible chicken flocks (between three and six weeks of age), the disease is responsible for severe losses due to impaired growth and death, and from excessive condemnation of carcasses because of skeletal muscular hemorrhages (6). Susceptible chickens less than three weeks of age do not exhibit clinical signs (1) but have a subclinical infection characterized by microscopic lesions in the bursa of Fabricius and immunosuppression (6). Two antigenically distinct serotypes designated as serotype 1 and serotype 2 have been recognized in the Europe (6) and USA (3). Several antigenically variant strains of serotype 1 IBDV have been described in the USA (3). These strains differ from classical serotype 1 strain in that they produce a very rapid bursal atrophy, but with minimal inflammatory response in three to four-week-old specific-pathogen-free (SPF) chickens. The virus was identified by reacting with a known anti-IBDV serum using any number of antigen antibody tests (7). The antigen-capture-enzyme-linked immunosorbent assay (AC-ELISA) was used to differentiate IBDV strains (4).

It has been reported that neutralizing monoclonal antibodies (MAbs) R63 and B69 can be used to differentiate IBDV strains into three groups. Field isolates of IBDV were primarily placed in group III, whereas the vaccine viruses tested were placed in groups I and II. The antigenicity of the viruses in these three groups did not correlate with cross-virus neutralization test (2). In another study, Snyder *et al.* (8) used a panel of two non-neutralizing and six neutralizing MAbs in AC-ELISA to examine the antigenicity of 1301 wild typesof IBDVs isolated from different poultry flocks throughout the USA.

In Saudi Arabia and many other countries, the disease picture of IBD is still unclear and requires further investigations (7). The present study was conducted to investigate suspected infectious bursal disease virus infection among broilers in the central part of Saudi Arabia, using AC-ELISA on bursal samples collected from diseased broiler flocks.

MATERIALS AND METHODS

Sample collection and preparation. A total of 142 bursa samples were collected from 15 commercial and nine backyard broiler flocks in central area of Saudi Arabia during 2007-2008. The age of birds ranged from two to eight weeks. The size of commercial flocks ranged for 5000 to 15000 birds and these flocks were vaccinated with classical strain of IBD vaccine at 14 days of age through drinking water. Number of birds in backyard flocks ranged from 200 to 300 and the vaccination program of these birds was not known. Eight freshly dead or severely ill birds from commercial flocks and three birds from backyard flocks were examined for postmortem lesions. Bursal samples were collected, chilled as quickly as possible and stored in frozen state for further processing. Each bursal sample was weighed and place in 1.5 mL Eppendorf tube. Antigen dilution buffer (diluted 1/20 in deionized water) was added to the sample in a ratio of 1 mL buffer per gram of bursa. Enough sand was added to the bursa after being cut to small pieces and the sample was ground using a pestle into a semihomogenous dense suspension. The tube was capped and the homogenate was frozen at -20°C. Before performing the assays, the homogenates were thawed, thoroughly mixed, and centrifuged at 1500 x g for 10 min. The thawed bursal supernatant of each sample was diluted 1:5 in antigen dilution buffer and used for virus detection and typing, using monoclonal antibodies (MAbs).

ELISA kit. The ProFLOK® IBD Ag capture test kit was used in the investigation. The kit was obtained from Synbiotics Europe (Lyon,France) and contained IBD screening plates pack (2 plates coated with monoclonal antibody "MAb" #8), IBD typing plates pack (1-MAb R63 coated plate, 1 MAb B69 coated plate and 1 MAb #10 coated plate), laboratory sand, antigen dilution buffer (10X), ready to use dilution buffer, IBD positive antiserum, goat anti-chicken horseradish peroxidase (HRP) conjugate, plate wash solution (20X), ABTS substrate solution and stop solution (5X).

Screening and differentiating AC-ELISA. The method described by Wu *et al*. (9) was followed for AC-EL ISA. Briefly, required wells of the IBDV MAb-coated plates were charged with antigen. Then bursa samples were added to the wells of MAb-coated plates. Positive samples were confirmed with rest of the MAb-coated plates (R63, B69 and #10 MAb-coated plates). The well strips were incubated overnight at 4°C, contents were discarded and washed three times with 1X wash buffer, followed by delivery of the IBDV positive serum and incubation of strips at room temperature. After three washings using the 1X wash buffer, HRP conjugate was added to each of the wells, followed by 30 min incubation at room temperature and three washings using the 1X wash buffer. Then ABTS peroxidase substrate was added, followed by 15 min incubation at room temperature and then diluted stop solution was added. The optical densities (OD) of the well strips were read at 405 nm in an ELISA reader (Flow Laboratories, England). Positive and negative control wells were considered, as described by the kit manufacturer. The OD values obtained from the plate reader were interpreted according to the kit supplier as followings: OD values $\geq$0.6 suggested the presence of sufficient IBD viral antigen to cause bursal damage (+), OD values $\leq$0.3 suggested the absence of IBD viral antigen (-), while OD values >0.3 and <0.6 were invalid and process was repeated. Differentiation between variant strains of IBDV was based on the pattern of reaction against the panel of monoclonal antibodies. Data thus collected were analyzed by Chi square test using Minitab software.

RESULTS

Infectious bursal disease in commercial and backyard poultry was suspected based on clinical symptoms and presence of pathological changes in the bursa of Fabricius at postmortem examination. Later, the IBD was confirmed using AC-ELISA. By using ELISA screening plates coated with MAbs, 61.23% (87 out of 142) bursal samples were found positive for IBD. Four different IBD typing plates (MAbs coated plates i.e., #8, B69, R63 and #10) were used for further differentiation of these positive bursal samples (Table 1). It was observed that Classic, GLS, E/Del and RS593 virus was detected by using MAbs #8. MAbs coated plates B69 were able to detect Classic virus type only. MAbs R63 coated plates were able to detect classic and E/Del virus types, whereas #10 MAbs coated plates detected Classic and GLS virus types (Table 1). Variant strains of IBDV were detected in broiler and backyard poulty bursal samples collected from birds younger than 21 days, while Classic viruses were not detected until four weeks of age.

Data analysis revealed that significantly higher samples (54.02%) contained traditional vaccinal strains compared to less pathogenic strains not used in vaccine preparation (29.89%; $\chi 2 = 4.287$; $P = 0.038$) and nontraditional and highly pathogenic strains of IBDV (16.09%; $\chi 2 = 13.484$; $P = 0.001$). however, nonsignificant difference was observed between less pathogenic strains not used in vaccine preparation and nontraditional highly pathogenic strains of IBDV (Table 2). The field isolates of IBDV were primarily placed in group III, whereas the vaccine viruses tested were placed in groups 1 and II.

DISCUSSION

In this study, clinical investigation indicated that IBD was a major disease affecting broiler production in the investigated farms and localities in Saudi Arabia. Being the main target of the virus, bursa of Fabricius was selected as the tissue for antigen-capture. ELISA (6). Of the screened samples by ACELISA, 87(61.23%) were positive for the presence of IBD viral antigens. The monoclonal antibody capture ELISA was developed by Lee and Lin (1992) to detect antibodies to IBDV in chicken sera and compared with conventional ELISA. It was found that the monoclonal ELISA assay had lower non-specific reaction than conventional ELISA. In the present study, it was observed that ACELISA with MAbs was successful in differentiating the very virulent IBDV (vvIBDV) phenotype from less pathogenic types (9).

In the present study, it was found that out of 87 ELISA-positive bursal samples, 54.02% represented traditional vaccinal viruses, 29.89% represented traditional non-vaccinal viruses, while 16.09% were identified as virulent and nontraditional viruses (vvIBDVs) not previously identified in the region (Table 2). It was also noticed that variant strains of IBDV were detected in broiler bursal samples collected from birds younger than 21 days, while Classic viruses were not detected until four weeks of age. This agrees with the findings of Jackwood and Sommer (4), who reported that new IBD viruses were detected in different places around the world using molecular technique or serology. This data also suggests that viruses continue to change and may circumvent the immune system of birds despite their vaccination against IBD (7).

It has been reported that MAbs R63 and B69 can be used to differentiate the IBDV strains tested into three groups (2). In the present study, the field isolates of IBDV were primarily placed in group III, whereas the vaccine viruses tested were placed in groups I and II. The antigenicity of the viruses in these three groups did not correlate with cross-virus neutralization test (2). In another study (8), a panel of two nonneutralizing and six neutralizing MAbs were used in AC-ELISA to examine the antigenicity of 1301 wild types of IBDVs isolated from different poultry flocks throughout the USA. Examination of these isolates with protective, neutralizing MAbs directed against the VP2 structural protein of IBDV showed that four antigenically distinct groups of serotype 1 IBDV could be separated on the basis of the presence of one or more MAbs defined, conformation-dependent and multivalent neutralizing sites (8).

Conclusively, the AC-ELISA carried out in this study exhibited excellent specificity and sensitivity for the detection and differentiation of IBDV antigens in bursal samples, making it a powerful tool for epidemiological and vaccine efficacy studies. In conclusion, this study revealed that new variant strains of IBDV were detected in the samples that have been tested in Saudi Arabia and to our knowledge this is the first report about the existence of these virus strains in commercial and backyard broiler flocks in this country.

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Table 1. Screening and differentiation of infectious bursal disease virus variants in broiler bursal samples using monoclonal antibodies.

Monoclonal antibody and AC-ELISA reaction*				Virus type
10	R63	B69	8	
+	+	+	+	Classic
+	-	-	+	GLS
-	+	-	+	E\Del
-	-	-	+	RS593

*Monoclonal antibodies were supplied as ELISAcoated plate strips with the ProFLOK IBD Ag Capture test kit (Synbiotics).

Table 2. Identification and differentiation of infectious bursal disease viruses in bursal samples collected from diseased broiler farms (n=87) using AC-ELISA assay.

Positive samples		Type of virus
%	Number	
54.02a	47	Traditional vaccinal viruses (same strains used in vaccine preparation)
29.89b	26	Traditional non-vaccinal viruses(less pathogenic viruses not used in vaccine preparation)
16.09b	14	Virulent viruses (non-traditional highly pathogenic viruses)

Values with different superscripts in a column differ significantly (P<0.05).

NOVEL IBD+ ELISA FOR THE DETECTION OF PROTECTIVE ANTIBODY TO IBD (VP2) VECTOR VACCINE

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SUMMARY

The novel ELISA kit (ProFLOK® IBD PLUS) was developed to detect protective antibody (VP2) to infectious bursal disease virus (IBDV). IBDV vector vaccines (HVT+IBD) vaccinated broiler flocks were challenged with the E/Del US variant strain at various ages. The novel IBD PLUS ELISA result was compared with commercial classic ELISA (ProFLOK IBD). IBD PLUS ELISA detected high level of VP2 antibody and provided good protection correlation against the challenged, whereas the classic IBD ELISA failed to detect the antibody response to VP2 protein and had a poor protection correlation.

INTRODUCTION

IBDV belongs to the genus *Avibirnavirus* of the family *Birnaviridae*, whose genome consists of two segments of double-stranded RNA. The disease is highly contagious and immunosuppressive in young chickens. Two serotypes of IBDV have been described. Serotype 1 viruses are pathogenic to chickens. Viruses within serotype 1 can differ markedly in their virulence and pathogenicity. Serotype 2 viruses are not pathogenic to chickens. Viral protein 2 (VP2) and VP3 are the major structural proteins, forming the outer and inner capsid of the virus respectively (4). The antigenic site responsible for the induction of neutralizing antibodies is within a minimal region, called the

variable domain of VP2 and is highly confirmation dependent (1,5). This site is also responsible for serotype specificity. VP3 is a group specific antigen that is recognized by non-neutralizing antibodies.

In young chicks, a quick onset of immunity after vaccination is critical, as after the decline of maternally derived antibodies (MDA), the chickens are at risk of infection. However, high titers of MDA interfere with the early vaccination against IBD with classical modified-live vaccines (MLVs) (intermediate and intermediate plus). Therefore the timing of IBD MLVs administration is crucial. HVT expressing the IBDV VP2 gene was proven to be safe and effective vector vaccine. It can be administered either *in ovo* or by the subcutaneous route in one-day old chicks, in the presence of high titers of MDA (2,3).

These studies were designed to examine the immune status of chickens after vaccination with HVT expressing IBD VP2 antigen utilizing ELISA and to evaluate the correlation of ELISA titer and challenge protection. Two ELISA kits were used in this study: Synbiotics classic ProFLOK IBD Ab test (classic IBD) were used tissue-culture derived antigens; the novel Synbiotics ELISA kit, ProFLOK IBD PLUS (IBD PLUS) was developed utilizing bursar derived antigens.

MATERIALS AND METHODS

Field study. Twenty to thirty serum samples were collected at 1, 2, 3, 4, 5, 6 and 7 weeks of age from Vaxxitek (HVT+IBD) vaccinated and non-vaccinated broiler flocks. At 3, 4, 5, 6 and 7 weeks of age, six bursa samples were collected. Classic IBD and IBD PLUS ELISAs were used to analyze the serology and IBD antigen capture ELISA (Synbiotics) was used for analyzing the IBD antigen in the bursa.

Experimental challenge studies. Chicks derived from 38 to 42 week old breeders (Ross 708 X Ross 708) were vaccinated with IBD vectored vaccines Vaxxitek HVT+IBD or Vectormune HVT+IBD; HVT vector only were administered as vaccination control. All of the vaccinations were administered full dose at 18 d of embryogenesis. At 7, 10, 14, 17, 21, and 28 d of age, 10 birds from each treatment group were challenged with $10^{3.5}$ EID50 of Var-E IBDV. Seven days post challenge they were euthanized, and bursal body weight ratios were determined. Bursae were homogenized and tested using antigen capture ELISA. Sera collected at one day and at IBDV challenge were assayed for the IBDV antibody by classic IBD and IBD PLUS ELISA.

RESULTS AND DISCUSSION

MDA played an important role in protecting young chicks from the field IBD challenge. An accurate measurement of MDA is very critical for managing chicks' health. In the field study, both classic IBD and IBD PLUS ELISAs were used to assess the MDA. For the non-vaccinated broiler flock, the classic ELISA suggests that at three weeks of age there is a trace amount of maternal antibody titer, whereas IBD PLUS indicates that at five weeks of age there is no antibody titer. The field outbreak of IBD occurred at five weeks of age when there was no IBD Plus titer detected. Data suggest that IBD PLUS kit provides an accurate immune status of broiler chicks.

In the Vaxxitek HVT-IBD vaccinated group, IBD PLUS demonstrated the active seroconversion at four weeks of age with high titer. However, the classic IBD test failed to detect antibody in HVT+IBD vaccinated chickens. The elevated antibody titer detected by classic IBD at six weeks of age is due to the field infection, since the unvaccinated group also showed similar results at six weeks of age. Therefore, classic IBD ELISA can not be used for the accurate measurement MDA or for monitoring HVT+IBD vector vaccines but it can be used to monitor natural infection in broilers.

In the experimental challenge study, compared to classic IBD test, the IBD PLUS test is superior for monitoring the decline of MDA in unvaccinated birds that correlated with a change in percent protection against the Var-E IBDV challenge administered at various times post hatch.

The novel IBD PLUS is more sensitive than the classic IBD ELISA in measuring an active antibody response to both Vectormune and Vaxxitek vaccines at 17 and 28 d post hatch. The antibody responses detected by IBD PLUS correlated with the onset of immunity as assessed by the IBDV challenge. The degree of protection against IBDV challenge at 17-21 d appeared to be the result of the combined effects of residual maternal antibody and the onset of vaccine induced active antibody responses as measured by IBD PLUS. At 28 d of age, an IBDV antibody response could be detected in both groups of vaccinates with IBD PLUS, but not with the classic IBD.

In conclusion, in both field and experimental challenge studies, IBD PLUS is superior to classic IBD for monitoring MDA and detects the active immunity elicited by HVT-IBD vector vaccines. The combined use of both ELISA kits allows for the differentiation of chickens vaccinated with vector vaccines versus birds naturally infected.

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TRACHEOBRONCHIAL ASPERGILLOSIS IN PULLETS

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SUMMARY

Aspergillosis is an important mycotic disease of birds, including poultry. It often presents in poultry in one of two situations. The first is as an acute disease in young chicks or poults and results in high morbidity and mortality. This is often referred to as "brooder pneumonia." The second is as a chronic disease in adults that results in a lower morbidity and mortality (2,5).

"Aspergillosis" typically indicates a respiratory infection, affecting mainly the lungs and air sacs. However, this disease can also be systemic, ophthalmic, encephalitic, osteolytic, and a dermatitis (5).

The present case documents aspergillosis caused by *Aspergillus fumigatus* and *Aspergillus flavus* and a zygomycosis in four to seven week old pullets in which the gross lesions were confined to the trachea and the primary bronchi.

CASE HISTORY

Two live and three dead layer-type pullets aged seven weeks were presented for necropsy at the Turlock branch of the California Animal Health and Food Safety Laboratories. The primary complaint was an increased mortality (peak of 0.225%/day) and gasping.

The birds were housed in multi-tiered, standard cages. The house was located on the west side of two houses that had been converted to open air manure sheds. A storm occurred the day prior to the sudden increase in mortality in which the wind was blowing across the manure pit and toward the pullet house. A modified-live Newcastle-bronchitis vaccine was administered via spray three to four days prior to the increased mortality.

This case was submitted as a follow-up to two previously inconclusive submissions from the same flock. The previous submissions, at age four wks, described a sudden increase in mortality without respiratory signs. The first submission consisted of eight significantly autolyzed carcasses diagnosed with pulmonary congestion. The second submission consisted of three live and two dead birds which showed pulmonary congestion and mild ascites. A possible fungal plaque was seen grossly in the tracheal mucosa of one bird. A definitive cause of the increased mortality was not identified in these two submissions.

Necropsy. The live birds exhibited depression and difficulty breathing. Upon gross examination, the trachea in three of the five birds was reddened in focal areas with thickened mucosa covered with a moderate amount of yellow, caseous exudate at the middle segment. In two of the five birds, the lumen of the bronchi was completely blocked with a yellow caseous exudate. No significant lesions were noted in the lungs, air sacs, or other internal organs.

Histopathologic examination. Samples of brain, nerve, esophagus, syrinx, turbinates, trachea, bronchi, lung, kidney, liver, spleen, and bursa of Fabricius were collected in 10% neutral buffered formalin, processed for paraffin embedding in a Tissue-Tek-VIP automatic processor, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E).

Sections of trachea showed a thickened mucosa, deciliation, and loss of the normal epithelial architecture. Moderate lymphocytic and mild heterophilic infiltrations as well as congestion were also seen. The sections of syrinx had similar changes as the trachea and had moderate numbers of lymphoid

follicles in the submucosa. Fibrinonecrotic exudate mixed with large numbers of segmented fungal hyphae completely blocked the lumen of the bronchi. There was also heterophilic and lymphocytic infiltrations and lymphofollicular formation in the bronchi. The lungs were congested with interstitial and perivascular edema. Also, there was a fibrinoheterophilic exudate in the lumen and lymphoid aggregates in the interstium. The turbinates showed a moderate accumulation of fibrinoheterophilic exudate mixed with moderate numbers of coccoid-shaped bacteria in the lumen. The bursa of Fabricius had mild lymphoid depletion in the medulla of only one section.

Microbiologic examination. Swabs of tracheal mucosa from four birds were inoculated on blood agar, MacConkey's agar, and Sabourad dextrose agar. Swabs of two livers were inoculated on blood agar and MacConkey's agar.

A. fumigatus was isolated in three of the four tracheal cultures; *A. flavus* was isolated in one of the four tracheal cultures; and a member of the class zygomycetes was isolated in one of the four tracheal cultures. All other cultures were unremarkable.

Auxiliary testing. All other tests, including virus isolation, immunology, and environmental cultures, were unremarkable.

DISCUSSION

A. fumigatus is the most common etiologic agent of aspergillosis (5), although several other *Aspergillus* spp. have been identified as etiologic agents of aspergillosis including *A. flavus* and *A. niger* (1,5,8). Though rare, dual mycotic infections with multiple *Aspergillus* spp. and/or zygomycetes have been documented (1,6,8) although the role of the zygomycetes is not well understood. In this case, there was a dual infection with *A. fumigatus* and *A. flavus* complicated with a zygomycetes.

Aspergillus spp. are ubiquitous organisms and are commonly identified in the litter and/or feed (2,5). Aerosol exposure is an important method of infection (2,5). In this case, open air plates as well as touch plates from various areas in the house were taken with no growth of any *Aspergillus* spp. Samples of the house debris and manure from the sheds near by were also submitted for fungal cultures and were unremarkable.

The most common clinical signs associated with aspergillosis are dyspnea, gasping, cyanosis, and hyperpnea (2,5). Although these signs are seen in other diseases such as infectious bronchitis or infectious laryngotracheitis, the difference is that birds with aspergillosis usually do not have any respiratory noises associated with the signs described above (2,5). Some cases of aspergillosis showed no signs and only presented with a sudden increase in mortality (3), while others show severe signs including central nervous system disorders (1). In the present case, gasping was not noted until a few weeks after the sudden increase in mortality.

Fungal plaques or nodules associated with *Aspergillus* spp. are typically found in the lung or air sacs (2,5). Occasionally, as was the situation in this case, fungal plaques can be found in the trachea, bronchi, and/or syrinx (2,3,4,5). One theory as to why this may occur in some cases is that spores of *A. flavus* are larger than *A. fumigatus* and therefore become lodge higher in the respiratory tract (5). This is a possible explanation for the location of the lesions in this case.

Reports of tracheal aspergillosis suggest that vaccines against infectious bursal disease, infectious bronchitis, and Newcastle disease given prior to infection may have contributed to the disease (3,4). In the present case, a modified-live Newcastle disease and infectious bronchitis vaccine were given via spray three to four days prior to the increased mortality which may have contributed to the occurrence of disease in this case. Other studies also indicate that aspergillosis is often a result of immunosuppression or immune-suppressive events (6,7). One report noted that infectious bursal disease significantly increased the severity of *A. flavus* infection (9). In the present case, there was no indication of infectious bursal disease or other immunosuppressive disorder.

The pathogenesis of the fungal infection, as seen in this case, is not fully understood. And the source of the aspergillosis and zygomycotic infection was never found. The storm that occurred one day prior to the sudden increase in mortality may be associated with the infection. However, it is likely that the infection was multifactorial.

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INTOXICATION OF NINE DAY OLD TURKEYS BY A COMBINED EXPOSURE TO ORAL SULFAMETHAZINE AND MONESIN

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In chickens, toxic effects of tiamuline, erythromycin, sulfachlorpyrazine, sulfamethazine, sulphadimethoxine, sulfaquinoxaline and oleandomycin have been described (3). In adult turkeys, intoxications with monensin at above normal use levels have been reported (9). Toxic effects have also been reported in 24 and 52 day old turkeys (11). Possible interaction between sulfamethazine and monensin in turkeys have also been reported in veterinary meetings (6,7). We present data from a field case and from a trial demonstrating toxic effects of sulfamethazine-monensin interaction in nine day old turkeys.

Field case. *Day 0*: A turkey starter feed medicated with 100 ppm of monensin (86 ppm according to subsequent analysis) and 55 ppm methyl bacitracin is delivered to the farm.

Day 1: 12,150 male turkeys are delivered and divided into three barns, 4000 in barn # 1, 4000 in barn #2 and 4150 in barn #3.

Day 4: Mortality reaches 3.58%, 3.75% and 4.63% respectively. Poults are submitted to the diagnostic lab.

Day 6: The farm is visited by the feed company consulting veterinarian. He notices that some poults are shaking their heads. Necropsy of seven poults is performed on the farm. Five did not show any lesions, one demonstrated some dehydration and another had lesions of airsacculitis. Treatment with sulfamethazine is recommended and initiated next day. For the first day 4 L of a stock solution of 25% sulfamethazine was administered via a medicator set at 1:128. The stock solution was then diluted to 12.5% for the next days.

Day 7: 625 poults are delivered to compensate for the abnormal initial mortality.

Day 10: Mortality increases to 66.03%, 35.08% and 40.51% respectively.

Day 11: The farm is visited by the feed company representative, the feed company consulting vet and the hatchery consulting veterinarian. Both veterinarians necropsied birds and noted lesions on the kidneys. The hatchery veterinarian suggested possible sulfamethazine-monensin intoxication. Both recommended to immediately stop the sulfamethazine water medication and poults are submitted to the diagnostic laboratory.

Day 17: Mortality has reached 82.33%, 60.15%, and 64.92% respectively. Mortality in the 625 replacement poults reached 4.32% at one week. These poults did not receive medication in the water.

Lab results from the first submission suggested that beak trimming and toe nail clipping may have been too severe. A slight opacity of the pericardium sac was observed in one bird and was associated to the presence of urates; gizzards were either empty or filled with wood shavings and gallbladders were dilated. There were no significant histological lesions. Lab results from the second submission showed that the ureters were blocked by urates, and the gizzards contained only wood shavings. The only histological lesions were multifocal vacuolisation in the cerebral trunk and cerebellum with some neuronal necrosis and vascular congestion with hypertrophic/reactive endothelial cells.

The fact that the untreated 625 birds escaped the high mortality strongly supports the hypothesis of a toxic interaction between sulfamethazine and monensin. On the first day of treatment, 1000g sulfamethazine was administered to 11,282 turkeys. Assuming an average weight of 140g, this is equivalent to 633 mg/kg BW for the first day and 316 mg/kg BW for the remaining. In Canada, sulfamethazine is not labeled for use in turkeys, it is labeled in chicken for the cecal coccidiosis. In the US it is labeled for use in turkeys at 117 to 287 mg/kg.

Experimental reproduction. The case study provided a natural experiment suggesting a possible interaction between sulfamethazine and monensin. However several questions remained unanswered. Lab results suggested possible dehydration. What was the role of dehydration in the process? The initial dose was way above label. Would use at a lower dosage such as the Canadian chicken label (~250mg/kg) have a similar effect? Thus an experimental reproduction was made on the case farm in the following flock. The objective of this experiment was to test the following hypotheses:

1. Use of sulfamethazine at Canadian label dose in combination with monensin is toxic to poults.
2. There is a dose effect.
3. The lack of water prior to treatment with sulfamethazine potentiates the toxic effect of sulfamethazine and monensin.

Six hundred and twelve male poults were divided into three groups of 204 and fed a diet containing 88 ppm monensin and 55 ppm of bacitracin. The rest of the flock was used as controls, unfortunately, due to technical difficulties with the feed mill the control diet contained lasalocid instead of monensin. At seven days of age, each group was randomly allocated to treatment as described in Table 1. On the third day treatments were stopped and ten subjects (five dead and five alive) selected from each of groups were submitted for complete necropsy.

Mortality data clearly show the toxic effect of combined exposure to monensin and sulfamethazine. There is also a dose effect. Indeed, mortality was 53% in the medicated group at labeled dosage vs 100% in the 25% stock solution medicated group. Finally, we note that the lack of water had a potentiating effect inducing a mortality of 91% in poults receiving sulfamethazine as labeled.

At necropsy, the three groups demonstrated the same lesions as described above. In the case of group #3, a slight plurifocal myocytolysis was observed in muscle sections. Values for blood hematocrit and brain sodium levels are presented in Table 1.

DISCUSSION

Typical sulfonamide intoxication produces a well described hemorrhagic syndrome. In turkeys monensin intoxication produces skeletal myopathy and cardiomyopathy. The lesions described in this case and brain sodium levels are similar to those observed in cases of sodium poisoning in turkey poults (2). The lesions observed in the muscles were suggestive of ionophore intoxication. Vitamin E deficiency also induces encephalomacia but with capillary thrombosis inducing ischemic necrosis of variable extent that was not observed in this case.

In turkeys, monensin toxicity is related to either abnormally high dosages or secondary to interaction with other substances as tiamulin (12), chloramphenicol (5), sulfachlorpyrazine (1). Tiamulin is a direct inhibitor of cytochrome P450 (9). In rats, elimination of monensin was reduced by 60% in the presence of tiamulin (8). In broiler chicken, monensin plus sulfadimidine causes a one day inhibition followed by a seven day induction of cytochrome P450 monooxygenase that is accompanied by a lowering of total antioxidant status (4).

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Table 1.

Group	Feed medication	Sulfamethazine	Mortality	Hematocrit (l/l)	Brain Sodium (ppm)
Control	Lasalocid 105ppm	none	36/3468 1%	n.d	
1	Monensin 88 ppm Bacitracin 55 ppm	Sulfamethazine as labelled	108/204 53%	0.38, 0.41	1846
2	Monensin 88 ppm Bacitracin 55 ppm	Sulfamethazine 25% stock solution on first day and then 12.5% stock solution medicator set at 1:128	204/204 100%	0.38, 0.36	1918
3	Monensin 88 ppm Bacitracin 55 ppm	Water withdrawn for 3 h and then sulfamethazine as labelled	184/204 90%	0.51, 0,42, 0.49	1813

EFFECT OF DIETARY VITAMIN E ON PLASMA OXIDATIVE STRESS IN HEALTHY AND *EIMERIA TENELLA* INFECTED BROILER CHICKS

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SUMMARY

We investigated the potential effect of dietary vitamin E on oxidative stress in broiler chicks fed with graded dietary vitamin E and infected with *Eimeria tenella*. For this, 96 day-old chicks were assigned into three treatments and fed on conventional diet supplemented with 0, 316 or 562 ppm of vitamin E premix. On the time of inoculation (d 21), half of the chicks in each treatment were inoculated with 4×10^4 sporulated oocysts of *Eimeria tenella* per bird, whereas the remaining chicks served as non-infected controls. Blood samples were taken and assayed for total antioxidant activity (TAA), lipid peroxidation (MDA) level and vitamin E. The oocyst shedding was also examined in all treatments. Results showed that TAA and vitamin E level in plasma were not affected by diet ($P>0.05$). The lowest level of MDA ($P<0.001$) was noticed in the chicks treated with 562 ppm of dietary vitamin E, but the difference between the chicks fed regular diet or 316 ppm dietary vitamin E was not significant ($P>0.05$). The oocyst shedding was the lowest in the chicks treated with 316 ppm dietary vitamin E ($P<0.001$), but there was no significant

difference between the other two diet treatments (P>0.05). It is concluded that addition of vitamin E at 316 ppm to broiler basal diet can improve cellular defense system against *E. tenella* infection without any effect on the plasma antioxidant status, but higher values may have adverse effect.

One of the major drawbacks of extensive use of anticoccidial drugs is the development of resistance, which has been described for all anticoccidial drugs introduced so far (12). In addition, it is growing concern about the chemical residues of anticoccidial drugs in meat and eggs, encouraging researchers to investigate alternative prevention and treatment strategies such as in-feed supplements.

Vitamin E is a lipid soluble antioxidant scavenging free radicals in a hydrophobic milieu and plays an important role in physiological actions. Among the four saturated forms, α-tocopherol is considered as the most biologically active and is generally added to poultry feeds as all-rac-α-tocopheryl acetate, at levels from 17 to 48 mg/kg (9). This ester is hydrolyzed after ingestion and absorbed through the small intestinal epithelium in its un-esterified form and then readily incorporated into cellular membranes where it promotes integrity by functioning as an antioxidant (6).

In spite of the wide marginal safety of vitamin E, it is documented that its high doses can have pro-oxidative effect on human lipoprotein (1). Therefore, this study was conducted to evaluate the effect of higher levels of dietary vitamin E supplements on the oocyst shedding of *E. tenella* and also to follow the alterations to plasma antioxidant and MDA levels in broiler chicks.

MATERIALS AND METHODS

Chicks and diets. This experiment was undertaken on a total of 96 unsexed one-day-old broiler chicks (Ross 308). Immediately after arrival, the chicks were randomly allotted into three diet treatments with 32 birds each. The chicks in treatment 1 received mash corn-soybean-based diet during the experiment and served as controls of the experimental birds were, but those in treatments 2 and 3 were fed similar diet supplemented with 316 or 562 ppm of vitamin E premix, respectively. On d 21, half randomly separated into treatments 4, 5 and 6, respectively, transferred to another house and inoculated with 4×10^4 sporulated oocysts of *E. tenella* per bird, whereas the remaining chicks were kept on their corresponding diets and served as non-infected controls. To exclude the unwanted infection, salinomycin Na was also added at 60 ppm to the diet of all treatments up to 17 d of age. The diet was formulated to meet the nutrient requirements of Ross chicks. Throughout the experiment, the birds were kept in wire-floored cages with constant lighting and free access to feed and water.

Experimental infection. At first, the parent Houghton strain of *E. tenella* was refreshed by two continuous propagations on three-week-old chicks in cage condition as per Chapman and Shirley (3) and then stored at 4°C until use. On d 21, each bird in treatments 1, 2 and 3 was inoculated via crop intubation with 4×10^4 sporulated oocysts of *E. tenella* in 1 mL of PBS, whereas the chicks in non-infected treatments were sham-inoculated with the same volume of PBS.

Oocyst shedding. Fecal samples were collected separately from all treatments every 12 h during five to eight d pi. Then, the mean daily oocyst shedding was determined by conventional McMaster technique, and expressed as the number of OPG per bird.

Blood collection. Blood samples were taken via brachial vein from eight birds in each treatment on d 8 pi, transferred into 5 mL sterile EDTA-K_3 containing tubes and immediately centrifuged. Then, the obtained plasma was aliquoted into microtubes and stored at -80ºC until use.

Biochemical analysis. TAA of plasma was determined by ferric reducing/antioxidant power (FRAP) assay as per the method described by Koracevic *et al.* (7). The MDA level was estimated according to the method of Placer *et al.* (13). Plasma vitamin E values were measured spectrophotometrically on each specimen according to Martinek's method (8).

Statistical analysis. The obtained data were submitted to one-way ANOVA using the Sigma State software. Values were compared using Tukey's post hoc test when passed the normality test and Dun's post hoc test in case of failure to pass the normality. The significance of differences between mean values was set at P<0.05.

RESULTS

There was not seen any sign of infection in non-infected treatments. The infected birds showed clinical signs without any mortality. However, recovery was observed in all infected chicks.

There was no oocyst observation on the day before inoculation. Regardless of the diet composition, the oocyst shedding started in all infected treatments from d 6 pi and reached to the peak on d 7 pi. Furthermore, the chicks in treatment 5 had significantly the least shedding value during six to eight d pi (P<0.001); whereas those in treatments 4 and 6 had comparable values (P>0.05).

The plasma levels of vitamin E, TAA and MDA in all experimental treatments were depicted in Table 1. As shown, a noticeable difference in the plasma level

of vitamin E was only found between treatments 3 and 6 fed on 562 ppm vitamin E diet ($P<0.001$). The plasma level of TAA was not affected by diet treatment ($P>0.05$), but infection in the chicks of treatments 4 and 6 caused a significant drop ($P<0.05$). The plasma level of MDA was not affected by infection, but it was significantly decreased by supplementation of diet with 562 ppm vitamin E both in infected and non-infected treatments ($P<0.001$).

DISCUSSION AND CONCLUSION

In the present study, the oocyst shedding started in all infected treatments from d 6 pi and reached to the peak on d 7 pi. This finding is consistent with Chapman and Shirley (3), who suggested seven d pi is the best time for collection of the oocysts from the ceca of broilers infected with *E. tenella*. Furthermore, the shedding value was significantly reduced by diet supplementation with 316 ppm but not 562 ppm vitamin E, suggesting that dietary vitamin E at 316 ppm could have a suppressive impact on the replication and consequently the severity of infection with *E. tenella*; whereas its higher doses may have some adverse effects on the cellular antioxidant defense system. This claim is supported by previous report of Yamamoto and Niki (14), where they demonstrated that high amounts of vitamin E could destruct intestinal cells through the increase of free radicals.

According to our results, almost all experimental treatments had comparable plasma levels of vitamin E. It seems that the level of vitamin E in the control diet is sufficient to make the maximum plasma levels. Besides, there is apparently a homeostatic system controlling the plasma levels of vitamin E (2). The observation that non-infected chicks treated with 562 ppm vitamin E had significantly the least plasma value is in agreement with what reported by Meydani *et al.* (10). They discussed that the absorption of vitamin E correlates negatively with increasing of its doses.

In the current study, the plasma level of TAA was only affected by infection in the chicks of treatments 4 and 6. It seems that the enterocyte antioxidant status against the parasite is independent of its plasma level. In this respect, there is some controversy about the changes in the plasma antioxidant status during infection with *E. tenella*. For example, Georgieva *et al.* (5) observed an increase in plasma catalase activity during infection with *E. tenella*, whereas Ersalan *et al.* (4) reported just the contrary. The evaluation of plasma MDA level showed that there was no significant difference between infected treatments and their corresponding controls ($P>0.05$), but dietary supplementation with 562 ppm of vitamin E caused a significant decrease in the plasma MDA levels. In this respect, Paşaoğlu *et al.* (11) discussed that changes of plasma MDA levels may be independent of their cellular levels.

With due attention to the results presented here, it may be concluded that addition of vitamin E at a rate of 316 ppm to broiler basal diet can improve cellular defense system against *E. tenella* infection without any effect on the plasma antioxidant status, but its higher values may have adverse effect. Nevertheless, further investigations should be made upon the entrocyte level of vitamin E in chickens to reveal the possible cellular target component in response to high vitamin E supplementation.

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

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Table 1. Effect of graded levels of dietary vitamin E on the plasma levels[1] of tocopherol, total antioxidant activity (TAA) and malondialdehyde (MDA).

		Parameters		
Treatment	**Description**	**Tocopherol (mg/dl)**	**TAA (mmol/l)**	**MDA (nmol/mL)**
1	(Non-Infected +0 ppm)	0.53±0.13	6.57±0.04^{AE}	1.56±0.65^{AB}
2	(Non-Infected +316 ppm)	0.45±0.03	6.65±0.03^{BCD}	1.25±0.36
3	(Non-Infected +562 ppm)	0.35±0.03^{A}	6.16±0.10	0.72±0.46^{B}
4	(Infected+0 ppm)	0.43±0.07	4.46±0.32^{AB}	1.25±0.14
5	(Infected+316 ppm)	0.43±0.11	4.67±0.19^{C}	1.23±0.63
6	(Infected+562 ppm)	0.58±0.15^{A}	4.45±0.19^{DE}	0.75±0.30^{A}

[1]Values represent means±SE for 8 samples per treatment.
$^{A-E}$Values in column with common upper-case superscript letters differ significantly (P<0.05).

MPI? OVER SCALD? DISPOSITION?

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SUMMARY

In 2008 a processing plant licensed by the Meat & Poultry Inspection (MPI) Branch of the California Department of Food and Agriculture processed quail for the first time. During the "startup" processing of quail, a MPI Branch Veterinary Medical Officer noted that the breast (pectoral) muscle of some quail had a blanched appearance suggestive of over scalding. Quail with blanched appearing breast muscle were submitted to California Animal Health & Food Safety Turlock Branch Lab for further workup. Efforts were made to adjust scald time and temperature to eliminate blanching with limited success.

On necropsy and microscopic examination of blanched versus non-blanched breast muscle, no difference was noted between the two areas, other than small numbers of lysed red-blood cells in the fascial surface of the muscle. No evidence of coagulative necrosis was found in the underlying skeletal muscle. To be classified as overscald, the muscle must be cooked through the level of the deep pectoral muscle. Based on these results, the blanching was determined to be not due to overscalding, and quail with blanching were passed as edible product.

Purpose and scope of MPI branch and Federal/California regulations pertaining to over-scalding/disposition will be described. Also, a controlled study using various scalding times of quail

will be presented to show at what temperature/time overscalding can occur with the appearance of gross and microscopic lesions of overscalding.

INTRODUCTION

Case of potential overscald is described in a new startup of processing quail at a plant under oversight of Meat & Poultry Inspection Branch (MPI). Description of MPI, regulation/disposition of overscald will be discussed. Controlled study to determine effects of scalding on processed quail and when condemnable overscald actual occurs will be presented.

MATERIALS AND METHODS

Case study. The Meat & Poultry Inspection (MPI) Branch of the California Department of Food and Agriculture (CDFA) licenses and inspects meat and poultry establishments that are exempt from United States Department of Agriculture (USDA) inspection, including 1) retail processors; 2) custom livestock slaughter plants; 3) retail poultry plants; 4) poultry plants processing rabbits, quail, pheasant, and partridge; and 5) poultry plants processing (<20,000/year) chickens, ducks, geese, guineas, squab, ratite, and turkeys (<5,000/year). MPI also licenses and inspects 1) renderers, 2) collection centers, 3) dead animal haulers, 4) pet food processors, 5) importers of meat and meat by products for pet food, and 6) pet food slaughter plants to prevent diversion of inedible animal tissue into human food and help assure wholesome pet food.

In 2008 a poultry plant in San Joaquin Valley of California began processing quail, chukar, and pheasants. This plant was already under MPI licensing and inspection because it processes ducks (<20,000/ year) and rabbits, and was exempt from USDA inspection. It also harvested rabbit blood and brain for incorporation into pharmaceuticals (for example, blood agar and thromboplastin). At this plant, there are seven Poultry Meat Inspectors (PMI) who are licensed but not employed by MPI. The plant and PMI are under the oversight of a MPI employed Veterinary Medical Officer (VMO).

During the initial processing of quail, the VMO observed that after picking, quail breast muscle was blanched and white in color, suggesting overscalding. Quail had gone through a scald tank at temperature of 140°F for 30 seconds. Reduction of scald temperature and/or time reduced the degree of blanching but did not eliminate it.

Overscald, according to the Code of Federal Regulations (1) is described as "breast muscle having a white or cooked appearance indicative of overscald." Overscald according to California Code of Regulations (2) is described similarly as "Carcasses of poultry that have been overscalded, resulting in cooked appearance of the flesh, shall be condemned."

To determine if blanching observed on quail extended into the breast muscle, birds with blanching were submitted to the California Animal Health and Food Safety Laboratory, Turlock Branch. On gross examination blanching only extended into the facial surface of superficial pectoral muscle. On microscopic examination only minor hemolysis of red blood cells was noted on the superficial surface histologically. Based on these results, a disposition was made that blanching did not extend into the muscle and that the muscle was not overscalded. The quail with the blanching over the breast muscle were therefore passed as edible tissue and not condemned.

To clarify disposition of carcasses that have a blanched or cooked appearance, USDA Food Safety and Inspection Services (FSIS) Directive (3) further differentiates disposition of overscald carcass from hard scald carcass. Cooking of the most superficial surface of the superficial pectoral (breast) muscle occurs in a hardscald carcass. This produces a whitening of that surface. In this directive, carcasses cooked to overscald in the poultry scalder to the level of the "deep" pectoral muscle (9 CFR 381.92) are to be condemned. Carcasses where the scalder only produced a whitening of the "superficial" pectoral muscle are to be passed. Since the blanching observed in quail was limited to the superficial surface of the superficial pectoral muscle, the disposition of passing carcass as edible tissue is in agreement with FSIS Directive 6100.3 Rev 1.

Controlled study. Quail were exposed to a scald bath and feather picking process at 140°F scald temperature for 30, 60, 90, 120 or 150 second intervals to determine the point at which overscald occurs, as defined by federal regulations and the effects of the scalder on processed quail. The normal scald temperature and time used at this plant is 140°F for 30 seconds. After processing, the birds were placed on ice and transported to the CAHFS, Turlock laboratory for evaluation. Each bird was photographed with skin intact and then again with the skin retracted back to expose the superficial pectoral muscles. Incisions were made into the superficial pectoral and deep pectoral (deep supracoracoid (4)) muscles to determine the extent of scalding as indicated by blanching or "cooked" muscle appearance. A small section of skin overlying the pectoral muscle and 1 to 2 cm sections of pectoral and deep pectoral muscle were removed from each bird and placed in formalin fixative. Tissues were processed for routine histology, cut at 3 µm, stained with hematoxylin and eosin, mounted, and cover slipped on glass slides.

RESULTS

Gross appearance. *Thirty-second interval*: Overlying skin intact and unremarkable. Facial surface of exposed pectoral muscle was moist and glistening. Cut surfaces of superficial pectoral and deep pectoral muscles were red and moist and did not appear blanched, or cooked.

Sixty-second interval: Skin intact, appeared unremarkable. Facial surface of pectoral muscle smooth, moist and glistening. Slight paleness to superficial pectoral muscle extending approximately 1 mm into muscle.

Ninety-second interval: Skin intact, although some bruising noted. Pale appearance to cut surface of superficial pectoral muscle extending approximately 5 mm into muscle.

One hundred twenty-second interval: Skin ragged and beginning to tear. Facial surface of pectoral muscle blanched and ragged appearing. Blanching extended deep into the superficial pectoral muscle, with a slight blanching observed in deep pectoral muscle.

One hundred fifty-second interval: Skin blanched, ragged, torn. Facial surface of superficial pectoral muscle pale, dimpled, torn in spots. Cut surface of superficial pectoral muscle blanched to level of deep pectoral (deep supracoracoid muscle).

Histologic results. Histological changes observed with increased exposure times to scalding were variable, and included lyses of red-blood cells in the capillaries of the dermis and hypodermis and coagulation of collagen fibers in the dermis. Swellings and fragmentation of skeletal muscle fibers and loss of cross striations were seen in skeletal muscle after 90 second exposure, becoming more pronounced as the time intervals increased. The epidermis was not present on any of the skin sections examined, having apparently sloughed during the initial scalding and feather picking process.

DISCUSSION

Exposure of quail carcass to scalding temperatures of 140°F for extended periods of time can result in tissue changes in muscle and skin that are apparent both grossly and microscopically. The visible changes are blanching, or a pale cooked appearance to the skin and underlying muscle, beginning with 90 seconds of exposure and progressing deeper into the pectoral muscle over time. The deep pectoral muscle was not affected until 120 seconds of scald exposure. Histologic changes were observed in both skin and muscle tissues beginning at 60 seconds of exposure; however, the changes were variable and difficult to quantify, and not as useful as visual inspection in evaluating the degree of scalding.

Based on gross and histological results, blanching and lesions of the deep pectoral muscle were not seen until 120 and 150 seconds. Since deep pectoral muscle was not involved at 30, 60 and 90 seconds, quail processed at these times could be passed as edible tissue, whereas quail processed at 120 and 150 seconds should be condemned for overscalding. These later times intervals are greater than four times the normally used scald time of 30 seconds at 140°F at this plant, suggesting that during normal processing, condemnable overscalding only occurs in extreme conditions.

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EGGSHELL APEX ABNORMALITIES (EAA) RELATED TO *MYCOPLASMA SYNOVIAE* INFECTION IN MULTI-AGE LAYER HENS: DIFFERENTIATION OF TWO STRAINS IN TRACHEAL AND OVIDUCT SPECIMENS

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ABSTRACT

The pathogenic role of *Mycoplasma synoviae* (MS) in the layer sector is still in debate. In this report we describe a clinical case of Eggshell Apex Abnormalities (EAA) that occurred in a layer farm in the North East of Italy. Most tracheal swabs, collected from several birds in different flocks on the farm, were positive for MS although the egg lesions were observed only in some flocks.

Our results demonstrate the presence of two different strains of MS, one apparently of tracheal origin and the other isolated from the oviduct; the latter could be the aetiological agent of EAA.

INTRODUCTION

Mycoplasma synoviae (MS) is considered an important pathogen for the poultry industry. It can cause problems such as airsacculitis and synovitis, mainly during intensive production. Its effect in the layer chicken industry is still a subject of investigation. Recently some Dutch and Italian researchers reported an unusual lesion of the apex of the eggshell, classified as Eggshell Apex Abnormalities (EAA) demonstrating a positive correlation between egg lesions and MS infection in the oviduct (1,2). In this study we report additional data to that previously published (1) concerning an outbreak of EAA in a multi-age Hy-line layer farm.

CASE REPORT

A brief description of the clinical case is given in order to better clarify and support our considerations.

When the farmer reported the presence of abnormal eggs the farm consisted of three different flocks. The abnormal eggs were found only in Flock 2 and 3 although showing different prevalence; 0.1-0.3% and 1.3-1.8 % respectively. In Flock 1 there was no evidence of abnormal eggs.

We planned a diagnostic protocol to apply to flocks on this farm in order to clarify the cause of EAA. This protocol included several examinations for evidence of infection with infectious bronchitis (IBV), *Mycoplasma gallisepticum* (MG), MS, and egg drop syndrome (EDS'76). Routine bacteriology was also carried out.

All the above tests were negative in Flock 1, whereas Flocks 2 and 3 were positive only for MS by serology and MS was detected in trachea and oviduct specimens both by culture and by PCR.

Following these laboratory results we decided to administer a treatment with tylosin (5g/L) in drinking water for five days, but only in Flock 3. Two days after the end of treatment no abnormal eggs were detected, suggesting the possible role of MS in the pathogenesis of EAA.

Unfortunately two weeks after the end of the treatment abnormal eggs were observed again although with a lower prevalence: 0.3-0.5%.

The prevalence of MS in oviducts correlated with the different prevalence of abnormal eggs between flocks; two out of six oviducts collected in Flock 2 were positive for MS (prevalence 0.1%) whereas in Flock 3 four out of six oviducts were MS positive (prevalence 1.3-1.8%).

Based on these findings we focused our attention on Flock 1, the only flock with normal eggs. Our goal was to correlate a possible positivity for MS with the appearance of abnormal eggs.

MATERIALS AND METHODS

The diagnostic protocol consisted of testing 10 blood samples and 10 tracheal swabs bi-weekly. The former were tested for the presence of antibodies to MS by ELISA and rapid serum agglutination; the latter were tested for MS by PCR. In this way we could establish when the birds became infected.

When Flock 1 became positive for MS we collected six birds, 10 tracheal swabs and 10 blood

samples from Flocks 1 and 3 (Flock 2 having gone for slaughter).

To increase the probability of sampling affected birds in the flock showing abnormal eggs (Flock 3) we collected samples from all hens present in the same cage where at least one abnormal egg was detected.

Selected animals were sacrificed and submitted for necropsy and samples of respiratory tract and oviduct were collected in order to clarify any correlation between egg lesions and MS positivity in these organs. These specimens were tested both by PCR and for mycoplasma isolation.

Once MS strains were obtained from trachea and oviduct a PCR, amplifying a conserved part of the *vlhA* gene, was performed as described by Hammond *et al.* (2009) (3). PCR products were sent for sequencing and the sequences were aligned and compared.

RESULTS AND DISCUSSION

Flock 1 became positive for MS after 10 weeks, although no evidence of abnormal eggs was observed during the entire cycle of production. The diagnostic protocol was applied only in Flocks 1 and 3. The analysis performed in Flock 1 demonstrated MS in the tracheal swabs but not in the oviducts of all six sacrificed animals. On the other hand we could demonstrate MS both in the tracheal and oviduct swabs from Flock 3, which had the highest prevalence of EAA within the farm.

The relationship between MS isolation from oviducts and the prevalence of EAA within flocks together with the improvement of production seen after treatment with tylosin (1), and the experimental results of Feberwee *et al.* (2009), suggest that the egg lesions are correlated with infection of the oviduct with MS. However in Flock 3 we found some hens positive for MS only in the trachea but not in the oviduct. Taking these findings together we hypothesized that two different strains of MS might exist, one with a tropism for the oviduct tissue, causing egg lesions of EAA and another one for the trachea. This hypothesis could explain the tracheal positivity for MS of Flock 1 without any evidence of EAA.

To explore our hypothesis we decided to sequence part of the *vlhA* gene of MS of some oviduct and tracheal strains in order to establish if any differences could exist between the strains.

A total of nine strains were investigated and, when the sequences were compared, some differences were observed with strains isolated from the oviducts presenting a 39 nucleotide deletion.

These data lend support to the hypothesis that two different strains of *Mycoplasma synoviae* may be present on the farm, one of them with tropism for trachea and the other one mainly for oviduct. In conclusion we have demonstrated that a different strain of MS may be present in the oviduct, which could be the cause of EAA lesions. Further strains need to be examined to confirm these findings.

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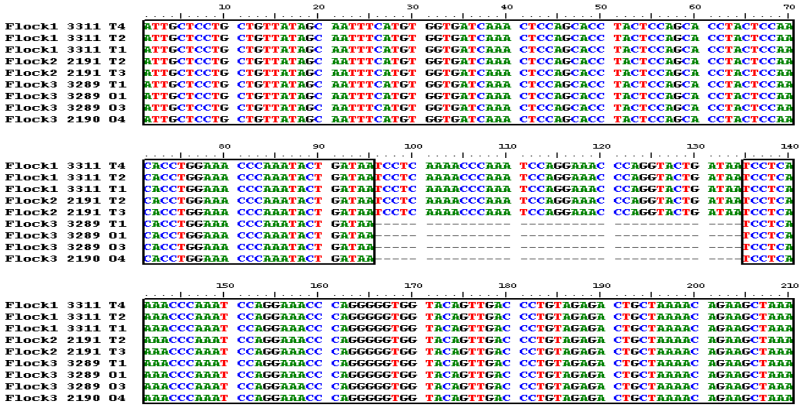
Figure 1. Alignment of partial vlhA sequences of the eight representative *Mycoplasma synoviae* strains identified in Flocks 1, 2 and 3. Samples are identified by flock number, the reference number of the laboratory, the organ of provenance: T (trachea specimens) or O (oviduct specimens) and the bird number.



STABILITY OF FLAA GENE IN *CAMPYLOBACTER COLI*

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SUMMARY

This study investigated the genetic stability of the locus flaA in *Campylobacter coli*. The locus has been proved to be variable in *Campylobacter jejuni*, even though data provided by different authors are often controversial.

We isolated two strains of *C. coli* from laying hens, and we typed them by PCR-RFLP of the flaA gene and by sequencing the corresponding PCR fragment. The strains underwent up to 50 passages *in vitro*. At the same time, we cultured the two strains up to 50 passages in presence of *Salmonella* Gallinarum to evaluate the effects of a competitor on the stability of flaA. On each passage, we performed PCR-RFLP, finding no differences in profiles if compared to the parent strains.

The flaA PCR fragments from the last passage were cloned and sequenced. The sequences from repeatedly passaged strains showed only point mutations, without significant differences with respect to the parent strains.

INTRODUCTION

Campylobacteriosis are among the most frequent food borne diseases in humans worldwide (1). *Campylobacter* spp. usually colonizes the intestine of warm-blooded animals. Poultry has been widely recognized as a reservoir for *Campylobacter jejuni* and *Campylobacter coli* (13), making consumption of chicken meat one of the most important routes for human infection (6).

The colonization of both human and avian intestine requires *Campylobacter* to express a number of factors controlling motility, adhesion to intestine wall, cellular invasion or toxin production (12). Some authors hypothesized that genetic variability could improve the fitness of *Campylobacter* and, consequentially, it capability to survive and to colonize dynamic environments such as guts of different hosts (10).

The full sequencing of the *C. jejuni* chromosome revealed hypervariable regions, which are physically associated to genes responsible for lipoligosaccharide biosynthesis, surface polysaccharide biosynthesis and flagellar modification (9). The latter group includes the flagellin genes, *flaA* and *flaB*. They are independent transcriptional units which are arranged in tandem (5). *flaA* and *flaB* share 95% sequence identity and it has been highlighted that *flaA* appears critical for motility, colonization and pathogenesis, while *flaB* represents a non-functional reservoir for genetic diversity, as recombination between *flaA* and *flaB* increases the variability of the former. Those reasons made *flaA* a good candidate for *C. jejuni* and *C. coli* genotyping (7).

This study was aimed to evaluate the variability of *flaA* from *C. coli in vitro*, by repeatedly subculturing two field strains and comparing PCR-RFLP profiles and sequences.

MATERIALS AND METHODS

Sixty cloacal swabs were collected from as many laying hens in two distinct Italian poultry farms, and they were processed to isolate *C. coli* by standard cultural methods (4). Identification was carried out by multiplex PCR according to Denis *et al*. (2) and all isolates were genotyped by PCR-RFLP according to Nachamkin *et al*. (8).

Two strains with different PCR-RFLP profiles and coming from different poultry farms were chosen and passaged in tryptic soy agar (TSA) plates supplemented with 5% defibrinated sheep blood up to 50 times. Both strains have also been subcultured on TSA supplemented with 5% defibrinated sheep blood in which 10^6 CFU of *Salmonella enterica* subsp. *enterica* ser. Gallinarum (*S.* Gallinarum) were included.

On each passage, PCR-RFLP genotyping was performed. PCR products from the first and the last passages have been cloned. Twelve clones for each experiment were sequenced, and nucleotide sequences were compared by the Mega 4.1 software (11).

RESULTS

Six out of the 60 samples resulted positive for *C. coli*. When we genotyped them by PCR-RFLP, we gathered two distinct profiles, C1 and C2. We chose two strain, BA29 and BA19, characterized by the profiles C1 and C2, respectively, and we subcultured them up to 50 times with and without a competitor, in this case *S.* Gallinarum. Following each passage, PCR-

RFLP of *flaA* was performed and we never found variation in profiles.

Additionally, we cloned the *flaA* amplicon from the first and the last passages of each series of subcultures, and twelve clones for each experiment were sequenced. The sequences of all clones from the last passage of strain BA19 were identical, while, after subculturing the same strain in presence of *S.* Gallinarum, we retrieved two distinct groups of sequences. Sequencing of clones deriving from the last passages of BA29 also returned two groups of sequences, either in presence and in absence of *S.* Gallinarum.

The results of comparisons among sequences from the first and the last passages are shown in Table 1.

Sequence comparison only showed point mutations and, following specific investigations, we excluded occurrences of recombination events. Assuming approximately 10-20 generations per passage, we calculated a substitution rate between $4x10^{-7}$ and $3x10^{-6}$ for the strain BA29, and not higher than 10^{-7} for the strain BA19.

DISCUSSION

The low number of substitutions does not allow a careful statistical analysis, but our data highlight that substitution rate of the *flaA* locus in *C. coli* is much higher than usual values, which have been assessed to be 10^{-10}-10^{-11} in *Escherichia coli*.

Furthermore, the competition stress seems to promote sequence variability as, when the two strains were subcultured together with *S.* Gallinarum, their *flaA* sequences exhibited more mutations in less passage.

Noteworthily, the strain BA19 showed a smaller substitution rate, insomuch that, lacking the competitor, we did not retrieve any sequence differing from initial.

On aggregate, our preliminary results suggest that some *C. coli* strains are more prone than other to variability, as well it has been reported for *C. jejuni* (3).

Since genetic variability may help *Campylobacter* spp. during colonization of gut, it is tempting to speculate that specific haplotypes may grant a greater variability and, consequently, specific advantages. Further studies should be addressed to verify those hypotheses.

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Table 1. Results of nucleotide sequence comparisons. Data are relative to the comparison of the sequences from last passage respect to sequences from parental strains.

Sequences[1]	Substitutions	Gaps	Transitions	Transversions
BA19(43)	0	0	0	0
BA19(50S)a[2]	1	0	1 (T→C)	0
BA19(50S)b[2]	1	1	1 (T→C)	0
BA29(44)a	2	0	2 (C→T)	0
BA29(44)b	1	0	1 (C→T)	0
BA29(30S)a[2]	5	0	1 (G→A) 2 (A→G) 1 (T→C) 1 (C→T)	0
BA29(30S)b[2]	2	0	1 (T→C) 1 (C→T)	0

[1]Names of sequences specify the strain (BA19 or BA29) and the passages from which the sequence has been obtained (reported parenthetically).

[2]S: *C. coli* grown in presence of *S.* Gallinarum.

AN AGENT BASED POULTRY DISEASE TRANSMISSION SIMULATION MODEL

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Models, either analytical or simulation types, have become more and more accepted as tools to understand disease transmission, to design control and/or surveillance programs and to perform risk analyses. We present the structure and processes of a computer simulation model of poultry production used to study the impact of biosecurity practices and the consequences of the uncertainty in biosecurity parameter estimates on disease transmission. The model simulates spread of a disease between poultry production units. It is spatially explicit and combines within production unit population dynamics, infectious disease dynamics and risk of transmission between units by means of aerosol, visitors, delivery trucks, etc.

The types of production simulated are laying operations, breeders and meat production (chicken and turkey). The industry practices reflect those within the province of Quebec, Canada; although these practices are very similar to those carried out elsewhere in North America.

We use an agent-based model (also referred to as individual-based model). Agent-based models are systems of interacting entities called agents (1). An "agent", is an entity defined by a set of variables describing the state of the agent and rules governing its behaviour and decisions. The model was implemented in the Python programming language (http://www.python.org/) using the Qt (http://qt.nokia.com/) application and user interface framework. Python and Qt allows for cross platform (Windows, Mac, Linux) use of the software. There are basically two types of agents, flocks and service suppliers.

SERVICE SUPPLIERS

The following service types are considered in the model: catching crews (starter pullets, broiler, turkey, layer); transport to slaughter; litter disposal; cleaning and disinfection crews; litter delivery; feed delivery; chick/poult delivery; live haul; field reps from feed company, hatchery, etc; veterinarians; utilities (gas, hydro); dead bird collection; random visitors such as salesmen, servicemen, unauthorized visitors, etc; vaccination, beak trimming crew, artificial insemination crew. Many of these suppliers deliver their services in a systematic and consistent way during the flock's production cycle i.e., hatching egg collection, chick and poult delivery, vaccinating and trimming, artificial insemination, feed, catchers, bird movement, litter disposal, cleaning crews, wood shaving bedding suppliers; and these cannot be considered random occurring events. Others provide services on an as needed basis (technical advisers, poultry veterinarian, fuel supplier, rendering) and some occur as random events (equipment sales and service representatives).

FLOCK

Each flock is characterized by a set of attributes (geographical location, bird type, size, and service supplier relationships). The flock undergoes a sequence of events that varies according to the specifics of bird type. Each production cycle begins with a delivery of litter if needed, feed by a feed delivery truck associated with a specific feed mill and a delivery of birds by the hatchery truck associated with a specific hatchery. During production, feed is delivered as needed based on feed consumption. Service providers may visit the farm as needed. The hatchery field rep will visit the barn once at 7 to 10 days. The feed mill rep will visit the farm at around 21 and 28 days. At the end of the production cycle, a bird catching crew will visit the barn to load the transport truck to the slaughterhouse. The litter is removed, and the barn may or may not be disinfected and remains empty for a given period of time after which a new cycle begins.

At the beginning of the simulation, one flock, selected at random, is infected with the disease of interest. The infectious process within a flock follows a deterministic SLIR (susceptible, latent, infected recovered) represented by a set of differential equations solved numerically. As the simulation progresses, the flock is visited by the different service providers (field reps, delivery trucks, etc). The disease can be transmitted by these service providers according to specific probabilities and level of biosecurity. As mortality reaches a threshold level, the production site will notify either the hatchery or the feed mill depending on the age of the birds to request a visit by their reps. The decision process regarding calling a veterinarian is also simulated.

If the option is activated, the disease can also be transmitted via alternative routes such as aerosol and vertical. The probability of infection by aerosol is modelled in a similar fashion as in the North American Disease Spread Model (NAADSM) (4) but taking wind direction into account. Vertical transmission depends on the disease status of the layer flock. The model can be initialized either from a data file or can generate the configuration at random.

Several modelling approaches have been exploited for the study of disease transmission between flocks especially for HPAI. Le Menach *et al.* (5) produced a stochastic mathematical model for farm-to-farm transmission that incorporates flock size and spatial contacts. A mathematical stochastic model combining within flock model, local area transmission and a network represented by a contact matrix was published by Truscott *et al.* (8). Sharkey *et al.* (7) developed a spatially explicit and stochastic model whereas Dent *et al.* (2) used social network analysis. Lewis *et al.* (6) used the NAADSM. Dorea *et al.* (3) presented a purely stochastic model. In all of these models, contact frequency and/or structure was based on probability distributions and ignored the fact that many contacts are the result of a systematic process often in direct relation to flock-size, and do not occur at random.

The proposed model takes into account the systematic processes involved as well as current knowledge from the scientific literature for any given disease, to offer a more realistic simulation reflecting how the commercial poultry industry functions in North America.

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SEVERE ORAL CANDIDIASIS IN COMMERCIAL TURKEYS

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ABSTRACT

Commercial 28-day-old male turkeys with severe oral lesions were submitted for examination with a presumptive diagnosis of mycotoxicosis. Oral lesions were characterized by erosions, ulcers and crusts extensively affecting the beak rim and the outer portions of the palate and tongue. Necropsy revealed no gross lesion other than mild aerosacculitis and entero-typhlitis. Histopathology of the oral tissues showed diffuse, severe ulcerative glossitis and stomatitis associated with myriads of 3-6 µm diameter, roundish, pale staining, thin-walled yeasts (*Candida* sp.) often arranged in short chains of pseudohyphae. Retrospective molecular investigations confirmed the involvement of *Candida albicans* in those lesions. The possible role of recent manual debeaking in these unusual *Candida* outbreak and the differential diagnosis of trichothecene toxicity are discussed.

INTRODUCTION

Candida species have a worldwide distribution and are part of the microflora of the healthy digestive system of humans, animals, and birds (3). Perturbance of the mucosal microflora, young age, concurrent infections and debilitation of the host can lead to candidiasis. Birds are particularly susceptible to oral and crop candidiasis, which resembles thrush in humans. In poultry production the occurrence of candidiasis is sporadic, but outbreaks can be costly. Diagnosis of candidiasis requires a multidisciplinary approach based primarily on conventional methods (direct microscopic examination of fresh smears, isolation, histopathology, etc.) often associated with molecular genetic techniques necessary to obtain more accurate and detailed insight (2).

MATERIALS AND METHODS

Animals. Seven 28-day-old commercial male turkeys out of a flock of 10000 were submitted for necropsy because of increased mortality and severe oral lesions compatible with a presumptive diagnosis of mycotoxicosis (trichothecene mycotoxins). Recent manual debeaking (second week of age) was reported as anamnestic data.

Histopathology. Samples of oral tissues (roof and floor of the buccal cavity and tongue), thymus, bursa of Fabricius, spleen, liver, intestine, and kidney were fixed in 10% buffered formalin and routinely included for histopathology. Four-micron sections were stained with hematoxylin and eosin and PAS.

PCR. DNA was retrospectively extracted from a selection of paraffin blocks from the archival tissue material shown to contain structures consistent with *Candida* on histopathology. Sterile razor blades were used to harvest 30 µg samples of tissue from the centres of the larger lesions. Samples were collected in sterile 1.5 mL tubes and dewaxed in 1 mL for 20 min, centrifuged at 1000 × *g* for three min, and washed twice with 100% ethanol. DNA extracted from the pellet by using a DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The identification of the yeasts was based on the amplification with universal primers (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2 5'-TCC TCCGCTTATTGATATGC-3') and sequence analysis of a region of the Internal Transcribed Spacer (ITS) of the rRNA gene as previously described (4). Negative and positive controls were included alongside the amplification reactions. Presence and size of the PCR products were assessed by agarose gel electrophoresis and ethidium bromide staining. PCR products of the expected length (218 bp) were purified from agarose gel slices using a QIAquick PCR purification kit (Qiagen) and sequenced using an ABI Prism 310 genetic analyser (Applied Biosystems, Foster City, California, USA). The sequence were submitted to the GenBank database (bankit1423376). The percentage of similarity with reference sequences was evaluated by BLAST search in the NCBI website.

RESULTS

Necropsy. All the birds showed unsatisfactory growth associated with severe oral lesions characterized by erosions, ulcers and crusts extensively affecting the upper beak rim and the outer portions of the palate and tongue. Signs of recent debeaking appeared evident in the seven birds. Mild entero-typhlitis with few oocysts in the cecal content was detected in two turkeys. Gizzards appeared normal. Mild fibrinous airsacculitis was observed.

Histopathology. Diffuse, severe ulcerative glossitis and stomatitis with abundant necrotic debris associated with myriads of 3-6 µm diameter, roundish, pale staining, thin-walled yeasts (consistent with *Candida* yeast) often arranged in short chains of pseudohyphae. Scattered aggregates of cocci were also found. Mild ballooning degeneration of the oral epithelium was focally observed in few sections. Mild to moderate lymphocytic depletion was found in all the spleen sections. As additional finding, mild, chronic typhlitis associated with coccidia at different developmental stages was observed in one section.

PCR and sequencing. Molecular analysis identified *Candida albicans* (similarity of 95%) as the yeast massively present in oral lesions.

DISCUSSION

The presumptive diagnosis of trichothecene mycotoxicosis was discarded as the other gross lesions typically associated with the oral involvement (yellow-tan, friable livers, swollen kidneys, urate deposits in the ureters, focal ulceration and inflammation of crop mucosa, and a thickened, rough lining in the gizzard) were lacking (1). Histopathological evaluation of all the oral samples from each bird revealed erosions and ulcers were constantly and massively colonized by structures consistent with blastoconidia and pseudohyphae of *Candida* as retrospectively confirmed by molecular analysis.

It was not possible to clearly assess which of the predisposing factors were primarily involved in this outbreak of oral candidiasis. Nevertheless, it is possible to hypothesize the recent manual debeaking played a major role. Matter-of-factly the oral lesions were significantly more severe in the upper beak, which was completely affected from the debeaking scar to the beak commissure including the whole oral roof. On the contrary, in the oral floor the lesions were limited to the beak and tongue margins. The turkeys had mild airsacculitis but it was not possible to ascertain whether they had received antibiotic treatment which could have enhanced *Candida* proliferation. Unfortunately, the isolation of this *Candida* strain was not performed. This could have been essential to characterize possible virulence factors related to the severity of this uncommon outbreak of candidiasis.

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PROTECTIVE EFFECT OF THREE INMNUNOGLOBULIN Y- BASED TREATMENTS IN BROILER CHICKENS AGAINST *EIMERIA ACERVULINA* INFECTION

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ABSTRACT

As an alternative strategy to control avian coccidiosis without using prophylactic medications, the objective of this study was to evaluate during the whole productive cycle of broiler chickens, the use of an immunoglobulin Y product (Supracox®). One of the groups received IgY´s powder in feed from d 1 to 42 (A), another IgY´s powder in feed from d 1 to 42 and liquid during three days during d 29 to 31 (B), and the last one IgY´s powder d 1 to 35 with salinomycin during d 36 to 42 (C). Upon orally infecting the broilers with a challenge of *Eimeria acervulina* at a concentration of 1 x 10^6 sporulated oocysts/mL at 29 d of age (four weeks of treatment), A and B treatments showed better reduction of fecal oocyst shedding (92.72% and 93.63% below control group) compared to group C (96.57% above control group). We conclude that the Immunoglobulin Y-based treatment is an effective option against a coccidia challenge infection.

INTRODUCTION

Avian coccidiosis is an intestinal disease caused by several distinct species of *Eimeria* protozoa and is the most economically significant parasitic infection in the poultry industry worldwide (2). Although prophylactic use of anti-coccidia feed additives has been the primary method of controlling avian coccidiosis, alternative control methods are needed due to increasing concerns with drug use and high cost of vaccines (4,5). Therefore, much interest has been devoted toward the development of drug-independent control strategies against coccidiosis (3,4,5). The use of egg yolk derived immunoglobulins as an alternative to antibiotic treatment for control in several animal species has been proved (1).

MATERIAL AND METHODS

Chickens and experimental design. One hundred male one-day-old commercial broilers were randomly assigned to five groups (20 birds/group). Each bird was vaccinated with NDV at the first day of age. Group 1 was administered IgY´s powder in feed (A), another one IgY´s powder in feed and liquid during three days (B) and the last one IgY´s powder with salinomycin (C); groups 4 and 5 were negative and positive controls. The animals were necropsied at d 7 post-challenge, the rest of each group at d 21 post-challenge to continue the fecal oocyst counts.

***Eimeria* and challenge.** Animals were orally inoculated with 1x10^6 sporulated wild-type strain of *E. acervulina* oocysts at four weeks old, seven, and 21 d post-challenge, chickens were necropsied and checked for characteristic acervulina lesions.

Immunoglobulin Y-based treatment. Group A was supplemented Supracox in feed from d 1 to 42 d of age, group B received the same plus three d (from one to three d post infection) Supracox powder in water.

Anticoccidial product. Group C was supplemented Supracox in feed from 1 to 35 d of age and salinomycin alone in feed from d 36 to 42. All groups received one last week feed without any treatment.

Parameters. Fecal oocyst counts from d 21 post-infection to d 49, each week. Table 1 shows the counts of oocyst per gram of feces.

RESULTS AND DISCUSSION

Immunoglobulin Y-based groups A and B reduced fecal oocyst shedding compared to the positive control group, 92.72 and 93.63 % less respectively; group C fecal oocyst shedding was 96.57% higher than positive control (Figure 1). Passive immunization of chickens with specific Ig-Y's is a good alternative against reduction of oocyst shedding. Lillehoj (6) studies showed significantly increased body weight gain and reduced fecal oocyst shedding between chickens given different concentrations of hyperimmune IgY egg yolk powder compared to control birds fed with non supplemented diet. In conclusion, this study demonstrates that Supracox is protective in at least one parameter during experimental coccidiosis.

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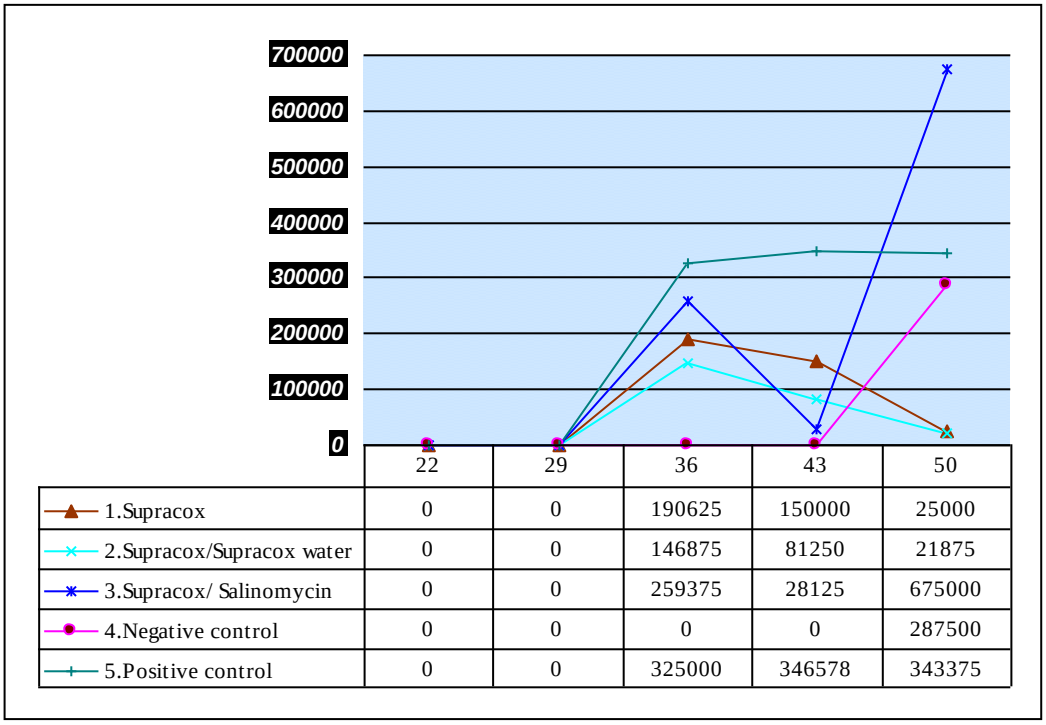
Figure 1. Fecal oocyst counts per gram of feces.



	22	29	36	43	50
1.Supracox	0	0	190625	150000	25000
2.Supracox/Supracox water	0	0	146875	81250	21875
3.Supracox/ Salinomycin	0	0	259375	28125	675000
4.Negative control	0	0	0	0	287500
5.Positive control	0	0	325000	346578	343375

SIMULTANEOUS TEMPORAL MONITORING OF IBDV, IBV, REO, AND NDV ANTIBODIES USING A LIQUID MICROSPHERE ARRAY

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Serum samples were collected in a random sampling of chickens from a Tyson broiler flock over a period of nine weeks beginning at d 4. The samples were simultaneously tested for IBDV, IBV, REO, and NDV antibodies using Luminex Multiplex Technology. This approach has the advantage of being able to analyze for multiple targets in approximately the same time as an ELISA can for one target. The testing showed a strong presence of maternal antibodies to IBDV and REO beginning at d 4 while there were none present for IBV or NDV.

APPLICATION OF METADATA ANALYSIS AND TEXT ANALYTICS TO EXAMINE AVIAN INFLUENZA RESEARCH

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SUMMARY

With a multitude of scientific literature available today statistical modeling and analysis can be applied to provide insight to help optimize future research. Utilization of text analytics and metadata analysis on avian influenza scientific literature can automatically group (cluster) and categorize articles into topics and sub-topics. A preliminary/training dataset was used containing literature spanning the last ten years focused on Avian Influenza research from or connected to China. In this paper, we use text analytics (statistical analysis on unstructured text data contained in documents) on this corpus of scientific documents to develop new insight amidst complexities of Avian Influenza.

INTRODUCTION

Avian influenza (AI) consists of 16 subtypes of hemagglutinin (HA) and nine subtypes of neuraminidase (NA) that produce numerous combinations. Each HA and NA combination represents a different subtype that can infect birds and, in some cases, even humans (2). Influenza A virus is classified under emerging infectious diseases (EID) as a re-emerging, or, resurging infection due to its evolution and ability to jump species and cause deadly pandemics (5). Re-emergence of EID can be attributed to "factors such as microbial evolutionary vigor, zoonotic encounters and environmental encroachment" (5).

Occurrences of highly pathogenic avian influence (HPAI) pandemics have resulted in significant losses to countries' economy, livelihoods, and human deaths (6). Since 2003 it is estimated that global HPAI outbreaks have cost billions (6). The main contributions to such economic and social impacts include: 1) HPAI is a zoonotic disease that can infect humans resulting in fatalities, 2) local outbreaks can cause loss in poultry production and livelihoods, 3) countries that experience large outbreaks suffer large control costs, and 4) migratory birds cause outbreaks that occur simultaneously throughout several countries (6). Such outbreaks prompt finding the best prevention protocols with respect to each country's poultry market and to track/predict outbreaks.

The International Telecommunication Union has estimated from 2005 to 2010 Internet users have doubled to over two billion (3). The Internet has provided a convenient means to access, gather, organize, process, and disseminate scientific data and literature to provide a basis in which to begin new or enhance ongoing research. Such applications include computer-based simulations as a way to try and predict epidemics and/or evaluate the effectiveness of control measures by modeling seasonal cases of influenza in subpopulations (1). The Global Epidemic and Mobility model (GLEaM) is an example of the application of collection of data to help predict future outbreaks of

influenza A (H1N1) (1). This is only one example of the multiple ways information accessible by the Internet can be analyzed.

Epidemiological data is generally gathered on a case-by-case base, causing discrete geographical and temporal data to be limiting factors, thereby forcing a constrained view of complex patterns that might be at play. Zoonotic infections continue to emerge as a global problem. This trend is likely to continue, possibly even accelerate. Avian influenza and other types of influenza infections continue to cause deleterious effects on both animals and man. As long as the virus persists, there remains the potential for pandemic. New ways of leveraging more and better data efficiently and economically must continue to be sought. Text analytics (data mining) has been proven as an effective means for developing new insight amidst complexity.

In the past, epidemiological data has consistently revealed travel of animals, people and equipment as elements of influenza virus transmission. Modern travel and trade patterns have increased the rate at which influenza and other viruses can circumvent what were previously isolated geographical locations. Local patterns of disease have historically been a means of determining transmission and predicting future risk. Disease has now become a global issue. Epidemiology remains in many instances, and in most countries and jurisdictions, a local or perhaps regionalized tool of discovery.

Text analytics (text mining) provides the capability of taking non-structured data from very disparate sources (reports, peer-reviewed journal articles, and epidemiological data) then uncovering associations and connectivity which might otherwise remain invisible. Isolate data is but one example where patterns over large geographical distances may be newly discovered. Our examination of existing data has consistently revealed the presence of similar strains of influenza virus in multiple countries. Other more complex patterns are also apparent, clearly indicating the need for more and detailed study.

MATERIALS AND METHODS

Data sources. A wide variety of sources were used to collect scientific literature on avian influenza pertaining specifically to China (whether research directly from or regarding thereto) over the past ten years. Databases used include: Ovid, PubMed, Agricola, CAB Abstracts, Biological Abstracts, Web of Science and Google Scholar. Through continuous collection currently over 8,500 article citations have been collected and will be incorporated into the developing model.

Data preparation. This preliminary study included 289 articles. Information regarding title, authors, author affiliations, publishing journals, publication dates, keywords and abstracts were pulled from each article. This information was imported into of SAS® Software where abstracts were modified by retaining important biological information.

Data description/analysis. From the 289, the number of authors ranged between 1 and 21, with a median of 7. There were several authors that appear as the lead author more than once, since the number of unique lead authors in the 289 papers was 168. The median number words in the abstracts was 188 (mean=183.1, 25th/75th percentiles = 137/251).

RESULTS AND DISCUSSION

In recent literature, the term "text analytics" has become a more accepted term over "text mining" in reference to the analysis of text. Text analytics literally means to analyze text, but it more generally refers to an organized frame-work for extracting usable and/or actionable information from large quantities of documents (unstructured text). Text mining is a method or collection of methods used to analyze text through pattern recognition (cluster, classification, data mining) techniques applied to collections of processed text. So, text analytics would generally refer to collections of techniques that provide ontology development, sentiment analysis and text mining to extract meaningful information and uncover patterns in large collections of unstructured text. For this paper, we focus on text mining for the purpose of clustering (unsupervised classification) and pattern recognition within a corpus of scientific documents related to avian influenza.

More specifically, extracting, spell-checking, topic processing and transforming the resulting text from the documents described in the previous section, we then used the many data mining tools available to explore concepts and relationships between documents (and authors and locations), create an ontology and to divide the documents into meaningful cohesive content groups. The clustering technique we used is applied not to the raw data (since it is unstructured text data) to low dimension data resulting from singular value decomposition (SVD) applied to the matrix formed by the "term-document" matrix, where "term" refers to individual words or phrases within the documents. An example of a term-document matrix is illustrated in Figure 1.

The numbers, within the individual cells in Figure 1, are the frequency counts of the ith term, i=1,...,m, within the jth document, j=1,...,n. Note: From Figure 1 it is easy to see now that the unstructured data (raw text from the documents) has now been transformed into a

structured form, where the documents are represented as vectors of term frequencies.

Before finding the Singular Value Decomposition (SVD), these counts are weighted using weights inversely proportion to the document frequency and dampened using appropriate transformations. Letting **A** be the m x n matrix formed by the numbers in the term-document matrix above, where m represents the number of unique terms (words or phrases) and n represents the number of documents in the corpus. Then the (SVD) is a decomposition of the matrix **A** into three new matrices.

$\mathbf{A = USV^T}$ where **U** is a matrix whose columns are the eigenvectors of the $\mathbf{AA^T}$ matrix, **S** is a matrix whose diagonal elements are the singular values of **A, V** is a matrix whose columns are the eigenvectors of the $\mathbf{A^TA}$ matrix and $\mathbf{V^T}$ is the transpose of **V**. Because of the above decompositions we can represent the vectors of information into a lower dimension by using the resulting "principal component-like" scores as our transformed data. This process converts our original unstructured data problem into a structured data problem of much lower dimension and the data can now be analyzed quantitatively using data mining techniques. For the sake of brevity, we only report that were able to divide the documents, using a clustering technique, and assign a theme to four of the clusters, which we label Clusters 1-4, and describe below.

Cluster 1- Surveillance [of flock migration and/or genetics (genotypes)]
Cluster 2- Molecular analysis
Cluster 3- Detection [techniques, i.e. PCR, ELISA]
Cluster 4- Health [Public health - affects on medical staffing, poultry workers]

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Figure 1. Example of a Term-Document Matrix

	Document							
Term	1	2	3	...	287	288	289	Total
surveillance	1	0	0	...	1	8	6	66
rt-pcr	1	1	4	...	0	5	8	57
. . .								
chicken/poultry	10	8	12	...	0	0	0	500

THE EFFECTS OF DIFFERENT LEVELS OF DDGS ON NECROTIC ENTERITIS DEVELOPMENT IN BROILER CHICKENS

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SUMMARY

The increased production of ethanol has lead to an increase in the amount of DDGS available for usage in animal feeds. The effect DDGS has on poultry gut health is relatively unknown. A goal of this experiment was to determine the effect two different DDGS inclusion rates have on necrotic enteritis development. In this experiment three diets were used, they were: control, 7.5% inclusion of DDGS and a 15% DDGS inclusion. These three diets were randomly assigned to 42 pens (14 pens/diet), birds in half of the pens (seven pens/diet) were challenged with an *Eimeria* cocktail on d 18. Birds were administered *C. perfringens* days 21-23. Fecal scoring for each pen was performed starting on d 21. Birds were necropsied and lesion scored on d 28. The results from this experiment implied that higher DDGS inclusion rates increased the incidence of necrotic enteritis.

INTRODUCTION

Necrotic Enteritis (NE) is a small intestine disorder that negatively impacts body weight gain, feed conversion, and the incidence of mortality of broiler chickens. NE is caused by *Clostridium perfringens* (CP), which is an anaerobic bacteria found in the normal intestinal flora of poultry (1). At low concentrations CP is not pathogenic. However, insults to the small intestine such as Coccidiosis or usage of certain feed ingredients can increase the population of CP that results in either clinical or sub-clinical forms of NE.

The broiler industry controls the incidence of NE by supplementing diets with feed-grade antibiotics. Due to the concern of the selection for antibiotic resistant bacteria, some U.S. broiler companies have reduced the usage of prophylactic antibiotics in diets. As a result, the incidence of Necrotic Enteritis has increased (2). Production losses will likely heighten if a greater number of broilers are provided antibiotic free diets. Emphasis should be placed on managerial and nutritional strategies to minimize the incidence of NE with the reduce usage of antibiotics in broiler production.

Ethanol production has increased exponentially in the U.S. and dried distillers grains with soluble (DDGS) are the co-product of ethanol production. DDGS is a feed ingredient for broilers that supplies a source for energy, amino acids, and phosphorus. In 2006, approximately 10 million metric tons of DDGS was available to the feed industry. The fiber composition of DDGS is predominately insoluble (42%) with a smaller amount being in the soluble form (0.7%) (3). DDGS is included in poultry diets at 5 to 15% with the inclusion rate being influenced by bird age, pelleting production rate, and economics.

Whitney *et al*. (4) conducted a disease challenge study with pigs fed diets containing DDGS. These researchers determined that a DDGS inclusion of 10% in the diet would reduce the incidence and severity of a *Lawsonia intercellularis* challenge compared with an antibiotic regimen. Pigs fed diets containing DDGS with no antibiotics had a similar proportion of ileal cells infected with *Lawsonia intercellularis* as compared with pigs fed diets supplemented with antibiotics. Pigs fed diets without DDGS and antibiotics had a higher proportion of ileal cells infected by *Lawsonia intercellularis.*

The efficacy of DDGS on reducing the incidence and severity of intestinal lesions after a NE challenge study has not been evaluated. The objective of this project was to determine the effects of DDGS on the incidence and severity of intestinal lesions after a NE challenge.

MATERIALS AND METHODS

Chicks and Management. Day-old broiler chicks were obtained from a commercial broiler company's hatchery and transported to the Auburn University Research Farm. The unsexed chicks were then randomly distributed into 48 battery cages (10 chicks per cage). Birds were fed a two-phase feeding program consisting of starter (from 0 to 14 d) and grower (from 15 to 28 d) diets. All of these diets lacked any sort of antibiotics. Prior to diet formulation, a sample of DDGS was analyzed for amino acid composition, amino acid digestibility, and metabolizable energy. These assay results were used in diet formulation.

Treatments. In this study there were six treatments, consisting of three diets with differing amounts of DDGS and if these diet treatment were challenged or not. The diets consisted of a control corn-soy diet – challenged and not challenged 7.5%

DDGS – challenged and not challenged, and 15% DDGS – challenged and not challenged. Each treatment will be represented by eight replicate pens.

Overall Challenge Scheme. At 18 d of age, broilers will be given a 1 mL gavage containing a mixture of *Eimeria* spp. From d 21 to 23, a 1 mL gavage of *Clostridium perfringens* will be administered. Fecal scoring will be performed starting four days post *Eimeria* cocktail challenge. Intestinal lesion scoring will be performed at necropsy, 10 days post *Eimeria* challenge (bird age 28 d) as described below.

Eimeria Challenge. Eimeria utilized in this study were – *E. acervulina* and *E. tenella.* The target amount to give to each bird was 200,000 sporulated oocytes for *E. acervulina* and 2,500 sporulated oocytes for *E. tenella.* In brief, feces were collected from birds that had been experimentally infected with either *E. acervulina* or *E. tenella* and placed into a 2.5% solution of potassium dichromate. This fecal slurry was then placed into a shaking water bath for 48h at 30°C. The slurry was then washed three times with distilled water and the number of sporulated oocytes was determined using a hemocytometer. From these counts it was possible to create an *Eimeria* spp solution where 1 mL of it was equivalent to the above mentioned target number of sporulated oocytes. On day 18, 1 mL of this *Eimeria* spp. solution was given to the challenged birds via oral gavage.

***Clostridium perfringens* Challenge.** The *C. perfringens* to be utilized in this experiment had been isolated from a bird that had been diagnosed with NE. After removing the bacterium from the -80°C freezer it was passed onto reduced tryptic soy agar containing 5% sheep blood and then incubated under anaerobic conditions (5%CO_2, 5%H and 90%N_2) at 37°C for 24h. After 24h each plate was checked for purity. If pure, a single colony was selected and used to inoculate 100 mL of brain heart infusion broth. The inoculated broth was then allowed to incubate in a shaker incubator for 24h at 37°C. After this time, the inoculated broth was diluted 1:100 to produce an approximately 10^6 CFU inoculum. One milliliter of this inoculum was given to the challenge treatment birds on the appropriate days (21-23). For each challenge day (21-23) fresh inoculum was made.

NE and intestinal lesions. Ne lesions were scored in the duodenum and jejunum using the scale described by Prescott *et al*. (5). Briefly, lesions were scored on a scale of 0 to 4, where 0 = no apparent lesions; 1 = thin friable intestine; 2 = focal necrosis, ulceration, or both; 3 = patchy necrosis; and 4 = severe extensive mucosal necrosis.

Fecal scoring was performed by counting the number of abnormal fecal droppings in the litter pan every 24 h. Determining the score is as follows – a 0 = no abnormal feces, 1 = 1-2 abnormal feces, 2 = 3-9 abnormal feces, 3 = 10-20 abnormal feces, 4 = no normal feces. Coccidosis lesions were scored according to the methods described by Johnson and Reid (6).

Data analysis. Data was analyzed using GLM with diet and challenge as main effects. If there was a significant difference ($P \leq 0.05$) means were separated using Tukeys Multiple Range Test.

RESULTS AND DISCUSSION

Fecal scores were recorded on d 22-28. Throughout the trial there were no meaningful differences between the treatments. The lone exception was between those that were challenged (high fecal scores) versus those that were not challenged (low to no fecal scores). Perhaps if more pens were utilized over this time frame a distinct pattern may emerge. That is a consideration for future research.

When the birds were necropsied at d 28 the challenged birds all exhibited typical signs of having or recovering from coccidiosis. Some of the challenge birds also exhibited lesion typical of NE. There was also a pattern based upon the amount of DDGS in the diet. Although this trend was not statistically significant (P=0.076) the high DDGS containing diet (15%) had a higher average NE score (0.25) compared to the diet containing 7.5% DDGS (0.20) or 0% DDGS (0.14). When diet x challenge NE results were analyzed there was still no statistical differences between NE based up on diet; however the trend observed with diet and NE was still noticed (Table 1). As can be observed the increase in NE lesions are even more distinct when diet type is further separated by challenge or no challenge. This implies that having lower levels of DDGS in the diets of chickens is actually better in reducing the incidence and severity of NE. Most of the research involving DDGS and intestinal health has involved swines. In pigs it is postulated that non starch polysaccharides (NSP) typically associated with DDGS is responsible for lowering the intestinal pH. This lowering of the pH is caused by volatile free fatty acids (VFFA) that are created by the digestion of NSP. This lowering of the pH has been associated with reducing CP growth in the human gut (7). DDGS also are high in dietary fiber. High fiber may cause additional mucin to be secreted by the epithelial cells; increased secretion would impair bacterial adhesion. However, as can be observed from our results neither one of these factors appear to play a role in reducing NE. It has been postulated that the undissociated VFFA (8) diffuse across the cell membrane and then dissociate. This results in a reduction of the cellular pH leading to cell damage. Perhaps damage to intestinal cells is occurring, which in turn is causing an immune response. This immune

response is causing CP to become more virulent thus leading to an increase in NE.

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Table 1. Challenge x Diet and Necrotic Enteritis Score.

Challenge	Diet	NE Scores
No	0% DDGS	0.04
No	7.5% DDGS	0.05
No	15% DDGS	0.05
Yes	0% DDGS	0.23
Yes	7.5% DDGS	0.36
Yes	15% DDGS	0.43

EFFECTS OF OREGANO ESSENTIAL OIL COMBINED WITH A NOVEL PREBIOTIC FIBER ON GROWTH AND LIVABILITY OF BROILERS ON A FARM WITH DERMATITIS CAUSED BY *CLOSTRIDIUM PERFRINGENS* TYPE A

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SUMMARY

A healthy gastrointestinal tract is critical for optimum broiler productivity. A key ingredient for gut health is a prebiotic. Prebiotics are nondigestable food that stimulates the growth of beneficial bacteria. Overgrowth of pathogens is less likely when a balanced gut microflora is established early. Oregano essential oil (OEO) is a natural feed flavor with potent antimicrobial activity against a wide range of pathogens, including *Clostridium*. This study investigated the effects of combining OEO and a novel prebiotic fiber on broiler performance on a farm with Clostridial dermatitis. The two control houses received in-feed anticoccidial program and antibiotic growth promoter. The two test houses received OEO (Regano® EX) and a new prebiotic fiber product (NutriFibe™ Complex). Results showed that the test houses performed as well as the control houses, demonstrating that this program is an effective rotational alternative for broilers. Clostridial dermatitis was not observed in the two test houses. One control house showed dermatitis at day 35, and this house had the highest death loss (3.1%). These results showed that Regano EX and NutriFibe Complex delivered excellent broiler performance and improved dermatitis control over the conventional program.

INTRODUCTION

The poultry industry is investigating natural products, including plant extracts because of their proven antimicrobial and antioxidant properties. Oregano essential oil (OEO) extracted from Greek oregano plants enhances appetite, improves digestion and supports intestinal health. University and independent research studies have shown that OEO and its active components, carvacrol and thymol, have potent antimicrobial activity against a wide variety of bacteria and fungi. OEO is pharmaceutical grade and "generally recognized as safe" (GRAS) by the United States Food and Drug Administration. It has no withdrawal period. OEO is environmentally friendly and leaves no harmful residues in manure, groundwater or soil. OEO has a very high level of antioxidant activity due to its phenol structure. Antioxidants are compounds that protect tissues and cells from oxidative damage. Antioxidants protect the integrity and health of gut epithelial cells, particularly during an inflammatory response. The USDA antioxidant database (http://www.ars.usda.gov/nutrientdata/ORAC) shows that oregano is far more potent that cranberries, blueberries, and other foods that are well known protective antioxidants.

To meet the nutritional needs of high performance birds, chicks need to develop a healthy, well-functioning gut early in life. A balanced gut microflora can maintain an effective barrier against overgrowth by enteric pathogens like *Clostridium perfringens*. NutriFibe Complex is a new product from Ralco Animal Health, designed to support gut health and promote the development of a balanced gut microflora. This product is a unique combination of prebiotic fiber, yeast cell walls (including beta glucans), and *Yucca schidigera* extract. The prebiotic fiber is selectively fermented by the beneficial bacteria, *Bifidobacterium* and *Lactobacillus*. These bacteria produce short chain fatty acids (SCFA) that reduce pH and promote maintenance of the gut mucosal barrier. The beta glucans support immune cell function and yucca extract is a source of natural saponins that are antiprotozoal and improve nutrient absorption. The objective of this trial was to determine if Regano EX and NutriFibe Complex improved health on a broiler operation in Texas with a history of dermatitis. The Ralco protocol was compared with the farm's anticoccidial and growth promoter. Weight gain, feed intake, death loss and the presence of dermatitis was measured.

MATERIALS AND METHODS

A four-house broiler operation with health issues, including dermatitis, was selected for this trial. Approximately 110,000 birds were divided into four houses on day one of age to begin the trial. All houses had built up litter. All birds received a standard corn-soy diet. The two control houses received the current rotation anticoccidial and growth promoter. In the two test houses, the anticoccidial and growth promoter

were replaced with Regano EX and NutriFibe Complex. Diets feed additives and feed changes are given below:

- Starter (d1-14): Control = Nicarb®, 3-nitro (22g), BMD® (50g); Test = Regano EX (0.88 lb/ton), NutriFibe Complex (0.5 lb/ton)
- Grower (d15-28): Control = Clinicox®, 3-nitro (34g), BMD (50g); Test = Regano EX (0.66 lb/ton), NutriFibe Complex (0.5 lb/ton)
- Finisher (d29-38): Control = Monteban®, 3-nitro (34g), BMD (50g); Test = Regano EX (0.60 lb/ton), NutriFibe Complex (0.75 lb/ton)
- Finisher (d39-46): Control = Stafac®, 3-nitro (22g); Test = NutriFibe Complex (0.75 lb/ton)
- Withdrawal (d47-49): Control and Test received plain feed

RESULTS

Results in Table 1 show that growth and livability of the test and control groups were similar. However, the conventional program did not control Clostridial dermatitis. Dermatitis was present starting on d 35 in one of two control houses. Mortality was 3.1% in this house. The test houses receiving Regano EX and NutriFibe Complex did not have dermatitis.

DISCUSSION

Awareness of the importance of gut health is increasing. Healthy epithelium allows for optimum nutrient uptake. A healthy and balanced microflora blocks enteric pathogens by competitive exclusion and supports intestinal immunity. Approximately 70% of the body's immune cells and tissues are located in the gut. This "gut associated lymphoid tissue" or GALT is the first line of defense against invading pathogens. The intestinal microflora influence maturation of the GALT shortly after hatch and provide important immune system regulatory signals.

One of the key ingredients in NutriFibe Complex is a proprietary prebiotic fiber which is preferentially metabolized by *Bifidobacterium* and *Lactobacillus.*This fermentation results in increased numbers of beneficial bacteria and the production of short chain fatty acids (SCFA). Butryic acid is about 30% of the total SCFA produced. Butyric acid, provides energy directly to the intestinal epithelial cell layer to maintain optimum nutrient absorption. In addition, SCFA reduce intestinal pH making the environment hostile for many pathogens. *In vivo* studies of of broiler gut flora showed that when chicks were fed this prebiotic fiber over a six week time period, *Lactobacillus* populations increased while *Clostridium* numbers were reduced.

Dermatitis is becoming increasingly prevalent in US broiler populations. This disease affects four to seven week old broilers and up to 10-25% of flocks may be affected. Clinical signs include poor appetite, skin lesions, edema, poor coordination, and leg weakness. Death loss may be 2-4% or higher. Bacterial pathogens associated with dermatitis include *Clostridium* spp., *E. coli*, and *Staphylococcus*. Coccidiosis control is important in preventing dermatitis, especially late cocci cycling. In this study, all four houses experienced dermatitis in the prior flock. Death loss was 4.5%. In this flock, one of two houses on conventional treatment had clinical signs of dermatitis and increased death loss. The two houses on the Ralco program did not show signs of dermatitis and death loss in both houses was similar. Prior efforts to control dermatitis on this farm had been unsuccessful. The results of this study showed that active management of gut health can improve the overall health and welfare of the broiler.

Table 1. Effects of NutriFibe™ Complex on growth, livability and dermatitis in broilers.

Group	House	Mortality (%)	Dermatitis?	Weight (lb)	Litter condition
Test	1	2.3	No		Fine
	2	2.9	No		Fine
	Mean	2.56		6.15	
Control	3	1.9	No		Fine
	4	3.1	Yes – at d 35		Fine
	Mean	2.50		6.16	

MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF OREGANO ESSENTIAL OIL FOR COMMON LIVESTOCK AND POULTRY PATHOGENS

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SUMMARY

The poultry industry is investigating oregano essential oil (OEO) because of its potent antimicrobial activity, which is attributed to its most abundant polyphenols, carvacrol and thymol. The antimicrobial activity of OEO has been demonstrated in different ways, and activity varies depending on the assay method, OEO source, and bacterial isolates tested. In this study, we used a validated microtiter assay to investigate the antibacterial activity of OEO. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for several livestock and poultry pathogens were determined, including *Escherichia coli*, *Salmonella* Enteritidis, *S.* Typhimurium, *Klebsiella pnemoniae*, and *Staphylococcus aureus*. Results showed that MIC and MBC ranged from 1.25-5.0 µg/mL, with the MBC equal to the MIC in all cases demonstrating bactericidal activity. Similar bactericidal action of OEO for *Clostridium perfringens* and *Candida albicans* has been shown previously. This study demonstrated that OEO in Regano® products has potent bactericidal activity for a wide range of pathogens and is a new tool to support gut health in broilers, layers, and turkeys.

INTRODUCTION

OEO is well known for its antimicrobial properties, as well as its antifungal and antioxidant actions. When harvested at the proper growth stage and steam extracted, OEO is a mixture of >30 different compounds. The major constituents, carvacrol (55-85%) and thymol (0-5-10%), have the most potent antimicrobial activity due to their phenolic structure. Mechanism studies have shown that carvacrol and thymol kill bacterial cells by altering the permeability of the cell membrane causing leakage of essential cations (1). Selectivity against gram-negative bacteria but with lesser activity against gram-positive *Lactobacillus* and *Bifidobacterium* has been observed (2).

Antimicrobials are often tested for their potency by evaluating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for different bacteria. The purpose of this investigation was to determine MIC and MBC of OEO for several livestock pathogens using a standardized microtiter protocol.

MATERIALS AND METHODS

MIC and MBC were performed for a wide range of livestock and poultry pathogens at the University of Minnesota Udder Health Laboratory using a microtiter assay method following Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (3). OEO (Ralco Animal Health, Marshall, MN) was prepared as a stock solution and serially diluted by two-fold dilutions in Mueller Hinton broth from 20 µg/mL to 0.039 µg/mL. Positive (no OEO) and negative (no bacteria) controls were included in each microtiter plate. Pure cultures of each bacterium were prepared on agar plates. A standard bacterial suspension was prepared (i.e. 0.5 McFarland standard), bacteria were added to microtiter plates, and plates were sealed and incubated at 37°C. Each microtiter plate was run in duplicate. After 18 h, wells were scored for bacterial growth (i.e. turbidity). The MIC was the lowest concentration of OEO showing no bacterial growth. After scoring for growth, one loopful of broth from each clear well was streaked onto a blood agar plate. Plates were incubated at 37°C and examined for bacterial growth after 18 h. The MBC was determined as the lowest OEO dilution showing no growth on plates.

RESULTS AND DISCUSSION

Table 1 shows that MIC for all pathogens ranged from 1.25 to 10.0 µg/mL. MBC ranged from 1.25 to 10 µg/mL, confirming prior reports that OEO is bactericidal for the bacteria tested. The killing activity of OEO has been attributed to the action of carvacrol on the bacterial membrane (1).

Several of the bacteria in this study are zoonotic pathogens (*Salmonella* spp., *E. coli* O157:H7, *L. monocytogenes*) or have zoonotic potential (methicillin-resistant *S. aureus*) (4). *S. aureus* is a

difficult pathogen to eliminate, whether it causes mastitis in dairy cows or skin, bone, or systemic infections in humans. Staphylococci sequester in fibrin-like clots, making elimination by antibiotics difficult. Methicillin-resistant *S. aureus* (MRSA) has been in the press recently because of its increasing spread in humans outside of the hospital setting and possible association with livestock and poultry. Infections caused by MRSA are difficult to cure because of its resistance to oral antibiotics. Results from this study and others (5) show that *S. aureus* is effectively killed by OEO at concentrations similar to other pathogens.

In summary, OEO is bactericidal for the tested bacterial pathogens at concentrations that are easily achieved in livestock and poultry feed. OEO has several advantages for use in livestock and other farmed species: It is GRAS (generally recognized as safe) by the FDA, has no withdrawal time, is effective against many gram-positive and gram-negative bacteria, and has low risk for development of bacterial resistance (6). In light of recent studies showing synergistic activity between OEO and several antibiotics (7), OEO may provide an avenue for reduction of antibiotic use in the poultry industry.

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Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oregano essential oil for livestock and poultry bacteria.

Bacteria	Source	MIC (µg/mL)	MBC (µg/mL)
E. coli K88	Swine	2.5	2.5
E. coli F88	Swine	2.5	2.5
E. coli O157:H7	Cattle	2.5	2.5
E. coli	Mastitis (cow)	2.5	2.5
E. coli	Mastitis (cow)	5	5
Klebsiella pneumoniae	Mastitis (cow)	5	5
Klebsiella pneumoniae	Mastitis (cow)	2.5	2.5
Streptococcus suis	Swine	5	5
Listeria monocytogenes	Cow	5	5
Salmonella typhimurium	Swine	5	5
Salmonella choleraesuis	Swine	5	5
Salmonella enteritidis	Poultry	5	5
Staphylococcus aureus	Mastitis (cow)	5	5
Streptococcus agalactiae	Mastitis (cow)	5	5
Streptococcus uberis	Mastitis (cow)	5	5
Pseudomonas aeruginosa	Human	10	10
Bacillus cereus	Environment	1.25	1.25

COMPARATIVE EVALUATION OF LYMPHOID DEPLETION OF BURSA OF FABRICIUS USING THE ADDL SYSTEM AND THE CONVENTIONAL SUBJECTIVE METHOD

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INTRODUCTION

The ADDL (Digital Evaluation of Lymphoid Depletion) system is a tool to analyze the lymphoid depletion of bursa of Fabricius, which allows the establishment of lesion scores through the digital analysis and the artificial neural networks. The ADDL was described by Moraes (3), who compared the depletion scores established by a histologist with those established by the ADDL system. At that time, however, the classificatory variability that existed among the scores established by the histologist was not quantified. For this reason, we decided to characterize this variability through the comparison between the depletion of bursae established by three different histologists, by the traditional methodology, and the depletions established by the ADDL.

MATERIALS AND METHODS

Bursa of Fabricius isolates. Fifty five bursa of Fabricius (BF) isolates were collected intact and kept in individual vials with 10% buffered formalin. These organs were collected in the previous experiment performed by Moraes *et al*. (2).

Preparation of slides. All BF isolates were processed for histological examination following the standard technique (1) and cut at the level of their major diameter in order to obtain the largest observation area.

Evaluations performed by the histologists. The isolates were first analyzed by the conventional subjective method, which is performed through the BF classification in depletion scores, from 1 to 5: Score 1 = < 25% depletion; Score 2 = 25% to 50% depletion; Score 3 = 50% to 75% depletion; Score 4 = 75% to 90% depletion; and Score 5 = >90% depletion (4).

The evaluations were performed by three avian histologists with different degrees of experience in this field. The slides should be evaluated by each histologist only once and randomly, but one histologist evaluated the isolates twice on different occasions (H1 and H2), with a four-month interval between them. Thus, it was possible to obtain four readings using the conventional method (H1, H2, H3 and H4).

Image capture. Each slide was divided into eight parts and two follicles for each octant were selected randomly for examination. The octants were numbered clockwise (I to VIII) and the follicles in ascending order (1 to 16).

Finally, digital photomicrographic images were taken under a 20X magnification with an OLYMPUS® C-7070 camera.

Evaluation performed using the ADDL system. The analysis procedure followed exactly the protocol mentioned by Moraes (3).

RESULTS

An analysis of the results of the lymphoid depletion averages obtained by the histologists (Table 1) revealed a great disparity among values. We observed a significant difference among almost all histologists (H1, H2, H3 and H4), including the two readings performed by the same histologist (H1 and H2). However, there was no significant difference between histologist 2 (H2) and histologist 4 (H4).

For the evaluation of the lymphoid depletion using the ADDL system, there was no significant difference among the analyses (Table 2).

DISCUSSION

The results of this experiment clearly characterize the difficulty in assessing the lymphoid depletion when it is performed in a conventional way. It is observed that the disagreement between histologists greatly undermines the reliability of these evaluations, especially when this variation involves readings of the same specialist (H1 and H2). This fact leads us to thinking that this classification actually occurs empirically, based solely on the experience of

the practitioner who performs it and subject to numerous uncontrolled factors. Unlike the qualitative classification, the quantitative classification leads the histologist to an uncomfortable classification and many resources are needed to hide this difficulty. These artifices are generally called "intermediário" or "intermédio" (intermediary or intermediate) and when related to lymphoid depletion scores, they are represented by scores 2, 3, and 4. The extreme scores (1 and 5) are easily classifiable, because they behave similarly to the qualitative classification.

The ADDL system generates a standardized evaluation of the lymphoid depletion, reflected in the lack of significant difference of the average scores obtained by the three different operators. The ADDL is not influenced by the experience of the practitioner who operates it, needing only the proper training of personnel to follow the proposed methodology, since operators who took part in this experiment are not histologists. Thus, the ADDL could be used in constant monitoring over time and the results could be compared scientifically.

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Table 1. Average values of lymphoid depletion of BF, obtained from the evaluation of three different histologists.

Histologists	Average scores of lymphoid depletion
H1	3.05455a
H2	3.65455b
H3	2.63636c
H4	3.47273b

H = histologist. Different letters in the column show significant difference.

Table 2. Average values of lymphoid depletion of BF, obtained from three different operators using the ADDL system.

ADDL system operator	Average scores of lymphoid depletion
DS1	3.06818a
DS2	3.05909a
DS3	3.06932a

DS = digital score. Different letters in the column show significant difference.

FIELD LYMPHOID ORGAN MORPHOMETRIC ASSESSMENT OF VECTOR HVT-IBDV (VAXXITEK®) VACCINATED BROILERS IN BOLIVIA

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SUMMARY

Morphometric evaluation of lymphoid organs in young birds allows the assessment of the flock immune status. The use of turkey herpes virus (HVT) vectors for infectious bursal disease virus (IBDV) transgenic VP2 expression is a current trend in IBDV control for commercial poultry operations. The objective was to compare the lymphoid organs morphometry in vector HVT-IBDV (VAXXITEK®) vaccinates versus live IBDV vaccinated broilers (two intermediate live) reared under Bolivia field conditions. A total of 435 broilers were evaluated (bursa, thymus and spleen weight, size, and indexes) between 21 and 37 d of age, comparing VAXXITEK versus live IBDV vaccinates. Results show bursal weight, size, and index were significantly higher *(P*<0.05) in the HVT-IBDV group. Similar results were observed for thymus and spleen, suggesting better lymphoid organs integrity. Flock immune status is vital for poultry health and performance, the use of vector vaccines avoiding valuable immature bursal B cells damage, generates measurable benefits in the bird's immune system.

Bolivia poultry industry confronts field challenges of immune-suppressive diseases including classical and variant strains of infectious bursal disease virus (IBDV) that affect the efficacy of vaccination programs and compromise performance. IBDV control is attempted using live and/or killed vaccines for the dams and/or offspring. A different approach is the immunization of chickens using viral vectors expressing the VP2 of the IBDV (2,3). The replication-competent herpesvirus vectors can express multiple antigens and both humoral and cellular immune response can be induced in the birds with the potential for long term protective immunity thanks to the persistent infection of the viral vector (2). Macroscopic evaluation of lymphoid organs size and relative weight in young birds allows the assessment of the flock immune status (4). The aim of this work was to compare the lymphoid organs morphometry in vector HVT-IBDV (VAXXITEK) vaccinates versus live IBDV vaccinated broilers in Bolivia.

MATERIALS AND METHODS

A total of 435 broilers obtained from farms vaccinated at one day of age with the HVT-IBDV vector vaccine or from live IBDV vaccinated farms were processed. Samples were obtained between 21 and 37 d old and divided for statistical analysis in four age groups as shown in Figure 1. The criteria to evaluate vaccination efficacy included body weight, bursa, thymus and spleen weight, size and indexes as previously reported (4). Non parametric Kruskal & Wallis test was applied at a *P*<005 significance level.

RESULTS AND DISCUSSION

Recently, experimental and field work has demonstrated the suitability of the vector HVT-IBDV vaccination for classical and variant IBDV control in meat type chickens (2,3). This trial describes the benefits of using a recombinant HVT-IBDV vector vaccine (VAXXITEK) for broiler IBDV vaccination under field conditions as indicated by lymphoid organs size, weight and morphometric indexes, demonstrating a novel tool for IBDV derived immunosuppression control. No differences in body weight were observed between treatments; nevertheless, bursal weight, size and index were significantly higher (*P*<0.05) in the HVT-IBDV group. Similar results were observed for thymus and spleen, indicating adequate lymphoid organs development in the vector vaccine groups. The relative size of the bursa strongly correlates with appropriate immunity in poultry (1). Figure 1 shows a consistent difference in the bursa/body weight index in favor of the birds vaccinated with the HVT-IBDV vaccine, suggesting lesser field IBDV effect on the vector vaccine group and variable degrees of bursal atrophy in the live vaccines birds. Additionally, the bursa/spleen ratio can be used as a field indicator of the immune status of the flock (4). In this trial significantly higher values were observed in the HVT-IBDV vector vaccinates beginning at 25 d of age, a higher bursa/spleen index that decreases over time in young birds suggests healthy bursa and spleen development. Flock immune status is vital for poultry health and

performance, the use of vector vaccines avoiding valuable immature bursal B cells damage, generates measurable benefits in the bird's immune system. Overall, these results demonstrate the suitability of HVT-IBDV vector one-day-old vaccination for broilers in Bolivia as indicated by lymphoid organs development, when compared with live traditional live vaccines programs.

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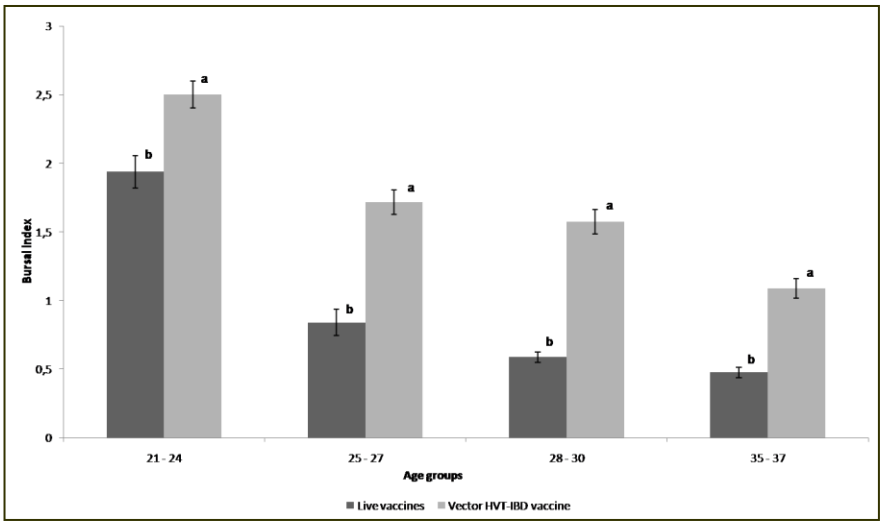
Figure 1. Bursa /body weight ratio in live versus HVT-IBDV vaccinated birds in Bolivia[A].



[A]Higher bursal indexes were obtained for the HVT-IBDV vaccinates in all the age groups.
[B]Different letters indicate statistical differences between treatments.

NUCLEOTIDE SEQUENCE DISCREPANCIES WITHIN THE GA STRAIN OF MAREK'S DISEASE VIRUS

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Comparative genomic analysis between nine Gallid herpesvirus type 2 strains has singled out the virulent (v) prototype strain GA as phylogenetically distant from other virulent pathotypes. Multiple amino acid alignments of otherwise highly conserved unique long (U_L) genes have indicated sequence discrepancies within 13 U_L genes (U_L6, U_L7, U_L12, U_L15, U_L16, U_L17, U_L18, U_L19, U_L26, U_L29, U_L37, U_L52, LORF11) and one gene within the repeat long region. Resequencing of these genes using both low and high passage isolates of GA (GA-p13 and GA-ATCC, respectively) has identified amino acid and sequence length polymorphisms in the original GA sequence relative to both GA passages and eight other sequenced GaHV-2 strains. Complete sequence agreement is obtained when the 14 genes of the two newly sequenced GA-passages are aligned with their counterparts from the genomes of eight other GaHV-2 strains, indicating the likelihood of sequencing errors within the original GA data.

Marek's disease is a highly contagious neoplastic disease of chickens caused by the alphaherpesvirus Gallid herpesvirus type 2 (GaHV-2). Control of this virus in modern poultry farms is of immense importance and vaccination programs have been in place since the early 1970s (16,25). Although initially very successful, these vaccination programs fail to induce sterilizing immunity and therefore hypervirulent strains have appeared over the last forty years (24). Because of this, there has been a growing interest in the comparative analysis of DNA sequences between virulent and vaccine strains. In the 1980s and 1990s, several regions of the MDV genome were sequenced (1,2,3,4,5,9,12,14, 17,18,19,20,26) but it wasn't until 2000 that the complete nucleotide sequences of two strains, GA (virulent) and Md5 (very virulent), were determined (11,23). The first completed genome of the prototype strain GA represented a composite of sequencing data from research workers around the globe. The bulk of the data was generated from *Bam*HI and *Eco*RI libraries (6,7) using Sanger-based sequencing with radioactive DNA separated on urea polyacrylamide gels and read by hand or "eye." Often this data was generated as part of a Ph.D. thesis or as a rite of passage during a graduate student's rotation in a prominent MDV laboratory. The end result was the complete sequence of the GA strain at unreported fold-coverage. This contrasts with the way sequencing data was generated for the Md5 strain. This group exclusively used automated DNA sequencers and random fractionated libraries to obtain a final DNA consensus representing on average a sixfold redundancy at each base pair (23) (Claudio Afonso, personal communication). Since then, seven strains of varying virulence (Md11, CVI988, RB-1B, CU-2, 584Ap80, C12/130-10 and C12/C130-15) have been sequenced (13,21,22) in order to identify genes and single nucleotide polymorphisms (SNPs) responsible for the pathotypes of the virus.

In examining nearly 200 open reading frames within eight GaHV-2 genomes, it became apparent that 14 genes within the genome of GA were heterogeneous relative to those of similar virulent strains (e.g. Md11 and RB-1B) as well as those in the attenuated strains CVI988, CU-2 and 584Ap80. In BLAST comparisons involving the other eight genomes, very few polymorphisms were noted within these 14 genes. Furthermore, computer software to identify SNPs routinely singled out GA as having the most synonymous and non-synonymous mutations of any of the GaHV-2 strains. Similarly, phlyogenetic trees (Fig.1) we have generated using the MAFFT program (10) with sequences encompassing the UL-IRL-IRS and partial US regions, as well as those generated by others (8), always grouped GA distantly from other virulent strains. Because of this, we decided to resequence various regions of the GA genome from both high and low passage isolates.

To achieve this, viral DNA was isolated from a low passage GA strain (GA-p13) previously passed in chicken embryo fibroblasts (a generous gift from Paul Coussens, Michigan State University) and a high passage isolate obtained from the American tissue culture collection (GA-ATCC) using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Primers were designed to flank 14 regions, mostly in the unique long region, that contained sequencing discrepancies. PCR products

were generated using standard conditions with Platinum Taq polymerase (Invitrogen, Carlsbad, CA) and processed for sequencing using the BigDye terminator cycle sequencing protocol and a model ABI-3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA).

We have identified regions within fourteen genes (U_L6, U_L7, U_L12, U_L15, U_L16, U_L17, U_L18, U_L19, U_L26, U_L29, U_L37, U_L52, LORF11 and RLORF1) in the genome of the original GA (accession number AF147806) isolate that contained amino acid and sequence length differences not found in the two newly sequenced GA isolates nor in the genomes of eight other GaHV-strains. Figure 2 contains the amino acid alignments of these regions. Since there is perfect sequence agreement between the two newly sequenced GA isolates and 8 other GaHV-2 strains within the differing regions of each gene, only one sequence (CONS) is aligned next to the original GA translation product. Sequence length differences were found in six GA genes: U_L6 (718 aa), U_L17 (743aa), U_L19 (1391aa), U_L26 (638aa), U_L52 (1075aa) and RLORF1 (239aa). Their counterparts in the other 8 genomes contained 722, 729, 1393, 663, 1074, and 198 amino acids, respectively. Inspection of the nucleotide multiple alignments (data not shown) give clues to the nature or potential causes for the differences among the 14 GA genes. Most differences could be attributed to di- tri- and polynucleotide misreads. Rarely were the differences based only on the inclusion or omission of a single nucleotide that was not part of a homopolymer stretch. Some differences were based on the absence of groups of nucleotides. Four nucleotides (ATGG) are missing at the 5' end of the U_L6 gene in the orginal GA sequencing data. All other U_L6 genes start with the sequence ATGGATGG. Three nucleotides (G_{45}, G_{94} and C_{100}) are missing from the U_L29 gene of GA. All of these "missed" nucleotides are part of dinucleotide pairs in the other U_L29 genes. Misreading stretches of repeating nucleotides is a common problem when analyzing sequencing gels.

Although it is easy to suggest that differences in the GA genes are the result of sequence misreading, we cannot definitively prove this without resequencing the *Bam*HI and *Eco*RI libraries used to generate the original GA data. The libraries are irretrievable. Given the fact that Sanger based sequencing has an error rate of 0.5-1.0% (15) and the existence of perfect sequence agreement between the two newly sequenced GA isolates and eight other GaHV-2 strains, it is highly likely that polymorphisms within these 14 genes in the original GA data are not real. However, it is not easy to explain the large insertion of 18 nucleotides present in U_L17 of GA. This is likely real.

Overall, this research has identified 14 genes within the original GA sequencing data that differ relative to sequenced PCR products generated from DNA taken from two independently obtained GA isolates. When these genes were resequenced using modern sequencing technologies and low and high passage GA DNA, data was generated which was identical to that found in the sequencing data of 8 other GaHV-2 genomes, both in amino acid composition and ORF lengths. The GA-p13 and GA-ATCC data has been submitted to GenBank, accession numbers XP886701 and XP886702.

It is likely that other sequence discrepancies within non-coding regions in the original GA data exist. Multiple alignments of nine sequenced GaHV-2 genomes have identified 209 GA polymorphisms. Designing PCR primers to resolve these differences or sequencing a complete genome of a new GA isolate has been contemplated and is planned for the future. However, since beginning sequencing of GaHV-2 strains using 454 Life Science pyrosequencing, we have generated a backlog of data from 10 additional genomes. The bottleneck is not in the generation of the data but in the resolution of data, especially homopolymer stretches. Although this modern type of sequencing technology can generate a 45-75 fold coverage (unpublished data) for an average alphaherpesviral genome, homopolymer stretches have to be resolved using PCR and classical Sanger based sequencing methods. This is extremely time consuming, but nothing compared to reading sequencing gels by hand, like the way they generated the original GA data.

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Figure 1. Unrooted phylogenetic tree of genomes of GaHV-2 virulent strains GA, Md5, Md11, RB-1B and attenuated strains CVI988, CU-2 and 584Ap80 based on the genomic segments TRL, UL, IRL and IRS and partial US sequences.

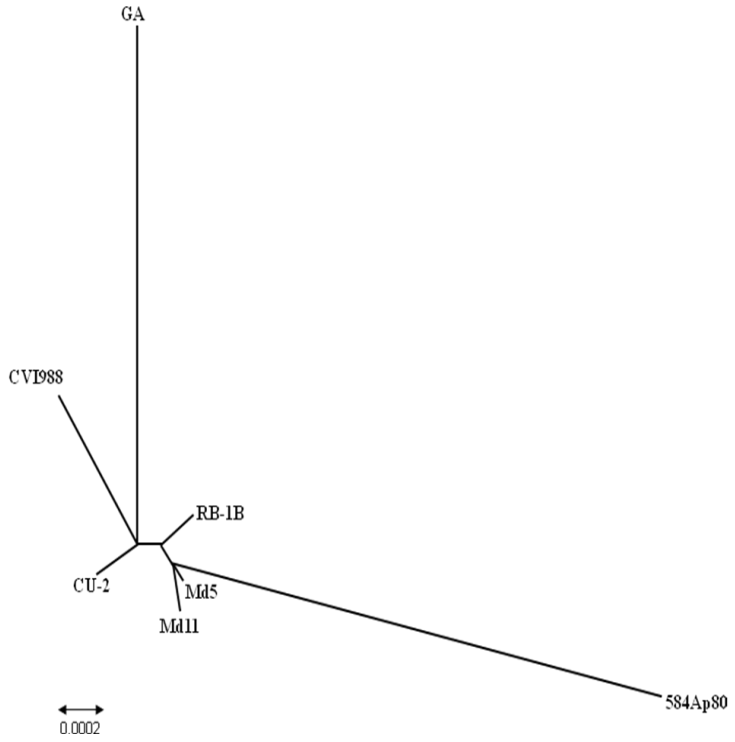

Figure 2. Multiple amino acid alignments of 14 GA ORFs that differ relative to their counterparts in the GaHV-2 genomes Md5, Md11, CVI988, RB-1B, CU-2, 584Ap80, C12/130-10 and C12/130-15. CONS represent the sequence found in all sequenced GaHV-2 including GA-13 and GA-ATCC. One exception is that U_L6 genes of C12/130-10 and C12/130-15 contain Ile at position 31. All others contain Thr[31]. Numbering above the alignment indicates the position in the alignment. Alignments with gaps contain numbering to the right in parenthesis indicating the coordinates of amino acids used in the alignment.

U_L6

```
                 *        20         *        40         *
CONS   : MDGYDRRAPYDLNSRKINHPDFESVMSQRDTFNNVSSKQCMENQWIVIHP (1-50)
GA     : MAMIRERLT--INSRKLIIRT--SNRLCRNEIPLHAHQTVYGDQWIVIHP (1-46)
```

U_L7

```
      260         *       280         *
CONS   : CSIVINKGLLNGAFQQKWLSDIICDWWHYGR
GA     : CSIVINKRLVEWSFPTKMVKYIICDWWHYGR
```

U_L12

```
         *        40         *        60
CONS   : SQILVGDEQSNSTKTKPYDEICENIVPNYTF
GA     : SQILVGMNNLTQLRQNLTMKYVKILYPNYTF
         *       120         *       140         *       160         *
180
CONS   :
ETEHILLDESRINNLSPSIHASRWCKMVESLTRLQANSELWHIFRQCLLTASSVKWSPNGTINTAGLITND
GA     :
ETVHILLDESRINNLSPSIMLLAGAKWCESLTRLQANSELWHIFRQMFADSIIGKVEPNGTINTAGHITTD
```

U_L15

```
        *       220         *       240
CONS  : QKMILMHATYFVTSVLLGDHAERAERLLRVA
GA    : QKMIFDACHLFCNFCFTWRSRRASERLLRVA
```

U_L16

```
               *        20         *        40         *        60
CONS  : MTTQRLKIPRSTRHQHSGRDCDGMDIEFVRRLNERIIIWRTLRAESRLVVMLALIALDSS
GA    : MTTQRLEDTKIYTPPALRKRLDGMDIEFVSRLNERYHHMAYTASRIPFGCMLALIALDSS
```

U_L17

```
       *        40         *        60         *        80         *
100
CONS  :IPDECLSAAG------
IDLTNLKDASPRYTTSVYSSLSVSVGVQIRQNATGLCSNWSNVYGEYVPSGALHS (30-94)
GA
:IPDECLIILMNVIGSGIDLTNLKDASPRILPVFTHLYLFPWAFKIGQNATGLCSNWSNLHREWRPSGALHS (30-
100)
             *       720         *       740
CONS  : RIEAVLSRLCRTNKMQQVLRPYVSEVYM*-------- : (702-729)
GA    : RIEAVLSRLCRTNKMQQVLRPYGFRGVYVTNPQHRY* : (708-743)
```

U_L18

```
       *        80         *       100
CONS  :TRFAAVITRALPGRMSAVVLGMGSIPNGLAL
GA    :TRFACRHHSCLAWPNERCSLGNGIYSNGLAL
```

U_L19

```
               *        20
CONS  : MAGCHCPPAGDCPPVAPCTF (1-20)
GA    : MAGCHCPGRGL-SAVAPCTF (1-19)
```

U_L26

```
           *       540         *       560         *       580
CONS  : VTSLQKEVERLNGGNLPISNAQSSYGVPNGMHAPVYYSYPPPGTHPTVSWPMGVE
GA    : VTSLKKKSNGLMEEIYRYQMHKVHMECPMGCMPQFITHTLLREHIPQFHGPWESE
      600         *       620         *       640
CONS  : PVSDPEAGRNVPITATISQERSDGIQKESIEQSRDTMNASA
GA    : PVFRSGGWSKCTNNCDHLSGAFRRNSEGKHRAITGYHEC*-
```

U_L29

```
               *        20         *        40
CONS  : MDGVGKSVKLCGGPIGYIYATPKCSVPVDELAILAAKSND (1-40)
GA    : MDGVGKSVKLCGGPIA-IYMRRPNVAFPWMNCDLAAKSND (1-39)
     640         *
CONS  :NIREGLG-DAN (640-649
GA    :NITRGVWGDAN (639-649)
```

U_L37

```
    1020         *
CONS  :QRVTADALVNI
GA    :QKSNSGSLVNI
```

U_L52

```
     280         *       300         *       320
CONS  :SVNPNPTGFLAADLTSFSRLSRFCCLSYYS-KGSVAIAFPS (280-319)
GA    :SVNPNTHGIFGCRFNVILVDYHVFVVEVTIPKALCPIAFPS (280-320)
```

LORF11

```
     120         *       140         *
CONS  :QALYNINCGPPWSLLGRIRTEVTVLRCVQVT
GA    :QALYNINCGASMVIIGKDKNRSHSLRCVQVT
```

RLORF1

```
      60         *        80         *       100         *       120
*       140         *       160
CONS  :DSAPGRPARPRTDPGSPERRPAGTT-CAG-
```

```
TGLERPPPRGARSVYSQSPARGSTAPTVVRAGPRVWERRRPRAPRRRGARGLPRGRRSRVALLGARGRAPH (60-
158)
GA    :DSAPGRRHGRGRTPVAPSG--AQRAPPAQGPGSSAHL-
RGGPVRSTHRAPRAAQRLQRSCARGRACGSDAVRAPHGAGGRGGCPGGAAPVLLCWGRGAALR (60-157)

                *       180         *       200         *       220         *
240
CONS  :LPGPAEADRGELFAAAMAGAGRPRPAGEDPRADGGERTPR*-----------------------------
------------ (159-198)
GA
:ISRAPPRLTEANSSRRQWRVPADRGLRARTPGRMGARGPLADVFFFLFAVEPLSVLSPASAPPPPPRGAPWGGSPS
PLKVSE* (158-239)
```