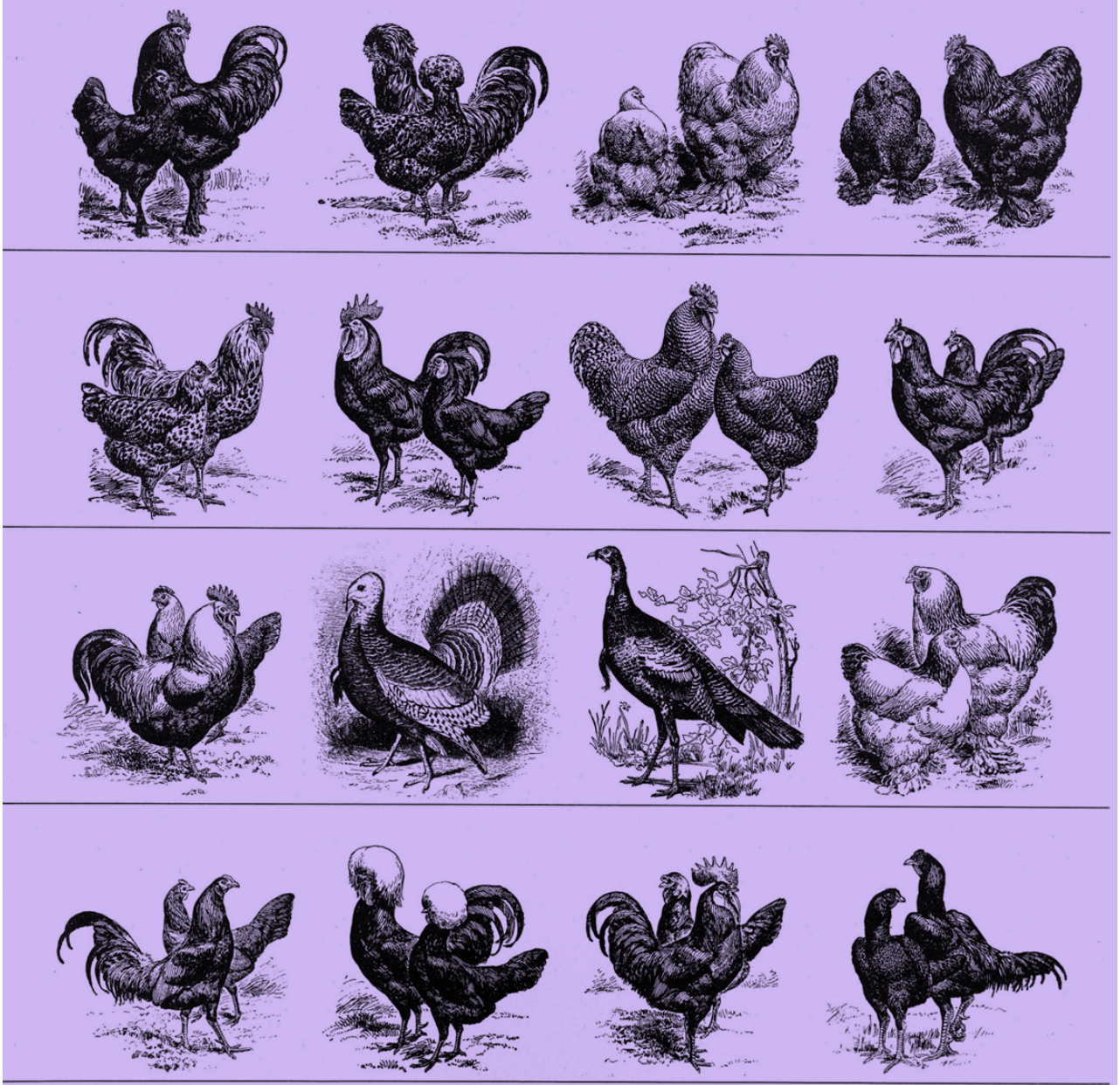
# PROCEEDINGS OF THE SIXTY-FOURTH WESTERN POULTRY DISEASE CONFERENCE

*March 22-25, 2015  Sacramento, CA*



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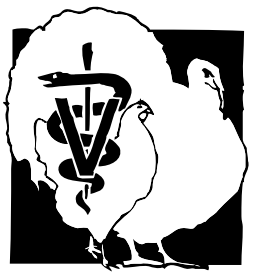

# 64TH WESTERN POULTRY DISEASE CONFERENCE DEDICATION

## BRUCE R. CHARLTON
## (1952 – 2014)

Bruce was born and raised in Loup City, NE. He joined the US Army soon after high school. Following his honorable service, he received a BS from the University of Maryland, College Park, MD, followed by a MS from Colorado State University, Fort Collins, CO, both in microbiology. Subsequently, Bruce received his DVM from Colorado State University in 1984. After a year in private practice in Nebraska, he headed west and accepted a position as a Veterinary Medical Officer in the Sacramento lab of the Veterinary Laboratory Services, California Department of Food and Agriculture. When the School of Veterinary Medicine at the University of California, Davis, assumed responsibility for the State's laboratory system, Bruce transferred to the California Animal Health and Food Safety Laboratory System (CAHFS) Turlock lab. At the time of his death, Bruce was a Professor of Clinical Diagnostic Microbiology and Branch Laboratory Chief at the CAHFS-Turlock lab.

He was a diplomate of both the American College of Poultry Veterinarians and the American College of Veterinary Microbiologists. He served on the Board of Directors of the American Association of Avian Pathologists (AAAP). Bruce was one of the first persons to characterize *Ornithobacterium rhinotracheale*. In addition, he developed molecular tests for the diagnosis of mycoplasma and salmonella. For these and other numerous accomplishments, Bruce was presented with the Poultry Scientist of the Year award by the Pacific Egg and Poultry Association in 2007. He is an author on more than 50 publications in refereed journals, 4 book chapters and more than 80 abstracts. Notably, he was the editor-in-charge of the 4th, 5th and 6th editions of AAAP's best-selling publication, the Avian Disease Manual.

Bruce died on May 26, 2014, following complications from surgery. He is survived by his wife of nearly 40 years, two sons, and three grandchildren. He will surely be missed for his never-ending smile, his jovial spirit and his "giggling" laugh. We proudly dedicate the 64th Western Poultry Disease Conference to Bruce R. Charlton.

# 64th WPDC SPECIAL RECOGNITION AWARD

# DAVID WILLOUGHBY

The Western Poultry Disease Conference (WPDC) is honored to present the 64th WPDC Special Recognition award to Dr. David Willoughby.

Dave was born and raised in Grundy Center, IA. He received his BS in mathematics from Stanford University in 1966. In possession of such a high-powered degree, Dave decided to join the US Marine Crop as an infantry officer. Following his honorable service, Dave and his beautiful bride, Laurie, headed back to Iowa where he obtain his DVM from Iowa State University in 1976. He spent 10 years in a food animal private practice in Minnesota. In 1986, Dave and Laurie decided it was time to move back to sunny California, where he went to work with the California Department of Food and Agriculture as a Veterinary Medical Officer. He retired from the CDFA in 2011.

In 1993, Dave enrolled in the Avian Medicine Residency program at the California Animal Health and Food Safety Laboratory System, Turlock branch lab, while still working for CDFA. Under the tutelage of Drs. Art Bickford, Bruce Charlton and George Cooper, Dave developed into a top-notch avian diagnostician. Upon completion of the 2-year residency, Dave continued working for CDFA, but now was the "go-to" person for all statewide issues pertaining to diseases of poultry. He worked closely with the California Poultry Federation and the California Poultry Health Board in guiding the industry on avian health concerns and regulatory issues, such as the National Poultry Improvement Plan.

Dave now spends his time with his family, fishing (but rarely catching), golfing (though some call it hacking), hiking, goose hunting (i.e., avian influenza surveillance), and whatever the boss wants. The 64th WPDC is honored to recognize Dr. Willoughby at this year's meeting.

# 64th WPDC CONTRIBUTORS LIST

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# SPECIAL ACKNOWLEDGMENTS

The 64th Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and to help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. More than 30 organizations, companies and individuals have once again given substantial financial support. Many companies and organizations, including some that also contribute financially, send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

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

Dr. Shahbaz Haq, Program Chair of the 64th WPDC would like to thank all the contributing presenters, and a special thank you to Dr. Richard Chin, and Dr. David Frame for their guidance and assistance with the organization of the scientific program. Warmest thanks to Tammy Simons for her outstanding work with the conference.

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

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

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# 64th WPDC PROCEEDINGS

*The **Proceedings** of the 64th Western Poultry Disease Conference are <u>not</u> refereed, but are presented as a service and a source of information to those attending the conference and to others who wish to gain some insight as to the information presented.* Copies of the 64th WPDC Proceedings are available in electronic format only.

**Copies of these Proceedings are available from:**

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| 14th WPDC – 1965 | B. Mayeda | R. Yamamoto | | |
| 15th WPDC – 1966 | R. Yamamoto | David S. Clark (1st sign of Contributors) | | |
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| 17th WPDC – 1968 | R. Balch | Richard McCapes | | |
| 18th WPDC – 1969 | R. McCapes | Dean C. Young | | |
| 19th WPDC – 1970<br>4th Poultry Health Sym. (PHS) | D. C. Young | W. J. Mathey | 1st combined WPDC & PHS | 1st listing of distinguished members |
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| 21st WPDC – 1972<br>6th PHS | R. Burdett | Marion Hammarlund | | |
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| 48th WPDC – 1999 | H. L. Shivaprasad | R. Keith McMillan | | |
| 49th WPDC – 2000 | R. K. McMillan | Patricia Wakenell | R. P. Chin | Ralph Cooper |
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| 51st WPDC – 2002 | K. Takeshita | Barbara Daft | Hiram Lasher | Bachoco S.A. de C.V. |
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| 57th WPDC – 2008 | B. Charlton | Rocio Crespo | A. S. Rosenwald* | B. Daft |
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| YEAR | PRESIDENT | PROGRAM CHAIR | DEDICATION | RECOGNITION |
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# MINUTES OF THE 63RD WPDC ANNUAL BUSINESS MEETING

In absence of President Cortes, Secretary-Treasurer, Dr. Richard P Chin, called the meeting to order on Friday, April 4, 2014, at 12:10 PM, at the Sheraton Buganvilias Resort, Puerto Vallarta, Mexico. There were 15 people in attendance at the time the meeting started.

## APPROVAL OF 62nd WPDC BUSINESS MEETING MINUTES

The minutes from the 62nd WPDC business meeting were reviewed and a motion was carried to approve them as recorded in the Proceedings of the 63rd WPDC.

## ANNOUNCEMENTS

Dr. Chin acknowledged all the contributors, in particular, Ceva Animal Health, which contributed at the Super Sponsor level. In addition, Dr. Chin acknowledged those at the Benefactor level, which included the American Association of Avian Pathologists. 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 recognized those who passed away this year. This included Dr. Ben Lucio, who we all know contributed significantly to poultry health in Mexico and the western region. (Note: Dr. George West, who had Lewy body dementia, passed away on April 2, 2014.)

## REPORT OF THE SECRETARY-TREASURER

Dr. R. Chin presented the Secretary-Treasurer report. The 62nd WPDC in Sacramento, CA, turned out to be a good one. Although there were only 143 registrants, there were contributions of $39,150 with an overall income of $71,785. We were able to keep the expenses to $59,190, thus leaving a net gain of $12,594. With this additional revenue, currently, WPDC has $111,304 in savings.

Contributions for this year's meeting (63rd WPDC) are, as expected, much lower at $26,550.

## REPORT OF THE PROCEEDINGS EDITOR

Dr. David Frame presented the Proceedings Editor report. This year, WPDC has joined the paperless world, and we are no longer printing a hard copy of the proceedings. The proceedings were supported by Lapisa.

## FUTURE MEETINGS

It was agreed to continue with the current rotation for meeting venues, with 3 different locations, i.e., Mexico, Canada and as yet undetermined. The meeting will continue to return to Sacramento every other year.

In 2015, the 64th WPDC and ACPV workshop will be back in Sacramento, March 22-25, 2015.

In 2016, the 65th WPDC and ACPV workshop will be in Vancouver, BC, Canada in late April. Dr. Chin had already obtained proposals from various hotels and narrowed it down to a few. After discussion of the proposals, it was agreed that the Marriott Vancouver Pinnacle Downtown was the first choice. Dr. Chin was instructed to continue the negotiation with the hotel and to sign a contract after approval by the WPDC Executive Committee.

## WPDC EXECUTIVE COMMITTEE

Dr. Chin reported that the WPDC Executive Committee nominated Dr. Susantha Gomis for Program Chair for the 65th WPDC in 2016. There were no other nominations and Dr. Gomis was elected unanimously as program chair-elect. Dr. Chin nominated the following officers for 2014-2015:

Program Chair:
President: Dr. Ernesto Soto
Past-President: Dr. Portia Cortes
Contributions Chair: Dr. Yan Ghazikhanian
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Richard Chin
Program Chair-elect: Dr. Susantha Gomis

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

## NEW BUSINESS

Dr. Chin stated that CE credits will be available online through ANECA. Registrants are to use the number on their name badge to download their certificate. There were no additional items for discussion.

Dr. Chin turned the presidency over to Dr. Ernesto Soto who acknowledged those who helped organize this year's meeting. He then adjourned the annual business meeting at 12:57 PM.

# CHARACTERIZATION OF IMMUNOGLOBULIN KNOCKOUT CHICKENS

K. Bolek and K. Klasing
University of California, Davis
One Shields Avenue, Davis, CA 95616

## SUMMARY

A study was conducted to characterize biological aspects of a new type of immunoglobulin (Ig) knockout chicken. Ig-deficient knockout chickens as well immunocompetent controls were given injections of keyhole limpet hemocyanin (KLH) at four, five, and six wk of age. Serum was collected at nine wk of age to determine the circulating IgA concentration. It was found that these Ig knockouts lack circulating IgA. Another experiment was conducted to examine the effect of KLH vaccination on body weight gain (BWG) and mucosal IgA production in these same knockouts. Ig knockouts and immunocompetent controls were given injections of KLH at four and six wk of age. BWG was significant from 35-43 d of age, with Ig knockout birds not given a vaccine having the highest BWG compared to all others. It was also found in this experiment that Ig knockout chickens also lack intestinal IgA. This has validates the use of these birds as a potential model to experimentally examine enteric pathogens and the nutritional cost of the adaptive immune response.

## INTRODUCTION

Ig knockout chickens have great potential as a research tool to examine avian adaptive immunity. Bursal ablation has been used numerous times to investigate aspects of avian immunity and infectious disease (1,6). However this is an imperfect method of produce B cell-deficient chickens. Bursal ablation tends to compromise the integrity of the cloaca. This can lead to leakage of rectal contents onto the birds, possibly affecting water balance and sanitation. The incomplete loss of B cells is also associated with this method. Even after bursal ablation occurs, a population of circulating B cells can still remain (5). A more appropriate model would be the use of genetic Ig knockout chickens. Ig knockouts would allow researchers answer questions relative to adaptive immunity without the previously mentioned complications.

Various aspects of infectious disease could be investigated as well as enteric pathogens. For example, the role of adaptive immunity in the gut against *Salmonella* Enteritidis is still somewhat unclear (2,3). This can be expanded further to other pathogens such as *E. coli* and *Campylobacter* species, which are important in the context of human health (7). These birds also have potential utility with regard to investigating the nutritional cost of immune defense. Thus, the objective of this experiment was to determine whether knockout chickens that have been shown to lack IgM and IgY in a previous study (8) would produce IgA in response to injection with keyhole limpet hemocyanin (KLH). A second objective was to determine the impact of Ig production on growth rate following vaccination.

## MATERIALS AND METHODS

**Experimental vaccination of birds.** Twenty-five birds, 10 immunocompetent controls and 15 Ig-deficient knockout birds were hatched and placed in floor pens. Controls were heterozygous for a knockout in the J segment of the Ig heavy chain while Ig-deficient birds were homozygous for the same knockout. Injections of 300 µg of KLH mixed 1:1 in complete Freund's adjuvant were administered intramuscularly to six Ig-deficient birds and eight controls at four wk of age. Boosters were given at five and six wk of age using incomplete Freud's adjuavant. At nine wk of age, blood was sampled from the brachial vein, placed in microcentrifuge tubes and spun at 5,000 x g for 15 min. Serum was collected and stored at -20° C for further analysis. A second group of birds using 13 Ig knockout birds and 16 wild type controls were placed in floor pens. Wild types did not possess the knockout. At four wk of age the birds were moved to individual cages. Injections of KLH were administered and prepared as previously described to six Ig-deficient birds and eight controls at four and six wk of age. Body weights were taken at four, five, six, and seven wk of age to determine body weight gain (BWG). At nine wk of age, jejunal mucosal scrapings were taken from each bird. Each sample was placed in six mL of PBS and spun at 5,000 x g for 30 min. Supernatant was collected and stored at -20° C for further analysis.

**Measurement of IgA.** An enzyme immunoassay plate was coated with mouse anti-chicken IgA (Bethyl Labs, Montgomery, TX). The plate was blocked with 3% wt/vol skim milk for one

h. Samples at both at 1:2000 and 1:4000 dilutions were placed on the plate. A third Antibody conjugated to HRP was incubated for one h and developed using TMB. OD was measured at a 450 nm wavelength.

**Statistical analysis.** Data were analyzed as a 2 x 2 factorial arrangement with genotype and vaccination as main effects by two-way ANOVA using JMP (SAS Institute, Cary, NC). Differences in BWG were considered significant at $P \leq 0.05$.

## RESULTS

**Growth performance**. Growth performance was affected significantly only during the period from 35-43 d of age (Table 1). An interaction between the vaccination status and genotype of the bird was present. Ig-deficient birds who did not receive a KLH vaccination and the WT were heaviest. All other groups displayed lower BWG and were not significantly different from one another.

**Tissue IgA concentrations.** It was found that Ig-deficient knockouts lacked detectable circulating IgA (Figure 1) as well as mucosal IgA (Table 1).

## DISCUSSION

Here we have confirmed that Ig knockout chickens lack circulating as well as intestinal IgA. This validates their use as a model for further determining the contribution of adaptive immunity in preventing infectious disease. Growth data from this experiment were unexpected. Activation of the immune system is associated with a nutritional cost (4), which one may expect to divert nutrients away from growth. Yet the controls did not experience different BWG compared to Ig-deficient birds when vaccinated with KLH. However, Ig-deficient birds did grow better overall from 35-42 d of age. The lack of a pronounced growth response may have been due to lack of immunological stimulation of innate immunity, which is the more nutritionally expensive arm of immunity. Indeed BWG from d 42-49 was not significantly different, suggesting little effect from KLH-stimulated immunoglobulin production. Further research must be done to further characterize the growth response in the birds.

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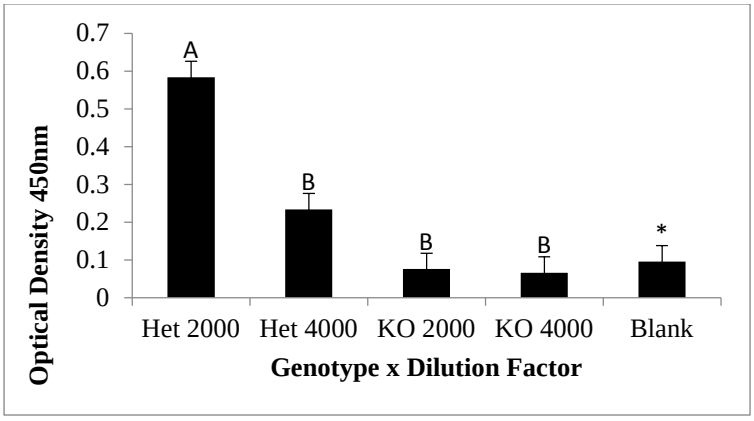
**Table 1.** Growth performance of birds injected with KLH at four and six wk of age.

| Genotype | Vaccination status[A] | BWG, 35-43 days of age[B] | Presence of Intestinal IgA |
|---|---|---|---|
| Wild Type | No | 77.95[b] | Detected |
| Wild Type | Yes | 98.90[ab] | Detected |
| Ig Knockout | No | 113.33[a] | None detected |
| Ig Knockout | Yes | 75.61[b] | None detected |

[A]300µg of KLH mixed 1:1 with complete Fruend's adjuvant was administered intramuscularly at four wk of age. A booster was administered at six wk of age were given using incomplete Freund's adjuvant.
[B]Numbers not sharing a common superscript differ significantly ($P \leq 0.05$)

**Figure 1.** Serum IgA concentrations at 9 weeks of age after vaccination with KLH mixed 1:1 with complete Fruend's adjuvant was administered intramuscularly at four wk of age. Boosters were administered at five and six wk of age. Horizontal axis displays genotype by dilution factor interaction of heterozygotes (Het) or Ig-deficient knockouts (KO). Values without common superscript differ significantly ($P \leq 0.001$)
[*]Blank used for comparison purposes and not included in statistical analysis



# SYNERGY OR INTERFERENCE OF A VELOGENIC NEWCASTLE DISEASE VIRUS CHALLENGE IN H9N2 AVIAN INFLUENZA-INFECTED CHICKENS ARE DOSE DEPENDENT

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## SUMMARY

Field observations indicate that the impact of velognic Newcastle disease (vND) is more severe in countries with concomitant circulation of low pathogenicity avian influenza (LPAI) virus, particularly H9N2. In our study we evaluated how the exposure of SPF chickens to a H9N2 challenge either favors or interferes with a subsequent vND infection and its transmission to sentinels. For this purpose, single vND and H9N2-vNDV challenges were performed with increasing doses of vND ($10^1$-$10^6$ $EID_{50}$). Co-infections resulted in the occurrence of a delayed but more severe clinical disease compared to single infections. Interestingly, at the lowest doses, vND replication was either favored, or halted by the H9N2 infection in a dose-dependent way.

## INTRODUCTION

Avian influenza (AI) and Newcastle disease virus (NDV) are two of the deadliest diseases of poultry. In the Middle East an ongoing epizootic of LPAI H9N2 has favored the occurrence of NDV-AI mixed infections in backyard farms and commercial layers, breeders, broilers and turkeys. In Israel, vaccination against velogenic NDV (vNDV) is mandatory (3) but even in the presence of good flock immunity, vaccinal breaks are often reported in association with H9N2 infections.

It has been speculated that H9N2 viruses can act as immunosuppressive agents (7) or cause an epithelial damage at the level of the respiratory tract, that in turn facilitates secondary bacterial or viral infections. In this study, we evaluated how an active H9N2 infection can affect the challenges of specific pathogen free (SPF) chickens with a vNDV.

## MATERIALS AND METHODS

In the first experiment, six groups of ten six wk old SPF chickens, were challenged with the genotype VII vNDV chicken/Israel/Maaleh-Hachamsha/998/2011, inoculating the animals via the oro-nasal route with 100µL of allantoic fluid containing $10^1$-$10^6$ $EID_{50}$.

In the second experiment, the same procedure was carried out three days after the birds had been challenged with $10^6$ $EID_{50}$ of the H9N2 A/chicken/Israel/1163/2011. Groups were named after the challenge virus and dose (i.e. NDV $10^1$-$10^6$ or H9-NDV $10^1$-$10^6$).

Challenge viruses were kindly supplied by Dr. Irit Davidson, Kimron Veterinary Institute, Israel.
In both experiments, two d after the vNDV challenge, five sentinels were introduced into each group and were cohoused with challenged birds for 18 d. On d two, four, six and eight post infection (pi) or contact (pc) all birds were sampled for the collection of tracheal and cloacal swabs. Clinical data were recorded daily and at the end of the trial blood was collected to assess seroconversion.

Viral RNA was extracted from 50 mL of phosphate-buffered saline containing a swab suspension using the Ambion MagMax-96 AI-ND Viral RNA Isolation kit for the automatic extractor. Isolated RNA was analyzed by rRT-PCR for the identification of the influenza A virus matrix gene using the published probes and primers from Spackman et al. (4). For the identification of the NDV fusion gene a rRT-PCR was carried out on the same RNA using a FAM probe (LproMGB) and a primer set (NDF and NDR) kindly provided by the Animal and Plant Health Agency, UK.

The hemagglutination inhibition test was carried out on sera using homologous antigens to the challenge viruses, according to the OIE procedure (6).

## RESULTS

In both the experiments, mortality of the challenged birds was dose-dependent while the severity of the disease among the sentinels did not correlate with the challenge dose. H9-NDV groups recorded higher mortality among challenged birds than NDV groups, at the doses of $10^3$-$10^6$ $EID_{50}$ (Fig. 1). At the lowest dose, the single NDV challenge

failed to either cause clinical signs or virus shedding by any of the animals, as it was confirmed by serology. On the other hand, the H9-NDV challenge with $10^1$ $EID_{50}$ could elicit overt clinical disease and shedding of the virus both by the challenged and sentinel animals. In this group, two sentinels became in fact severely sick and died.

Groups challenged with $10^2$ $EID_{50}$ differed greatly between the two experiments, as in the single infection two challenged birds and three sentinels died, while in the H9-NDV challenge, only one challenged bird died.

Regarding the onset of NDV tracheal shedding (Fig. 1), in the H9-NDV groups the virus could be detected from the trachea starting from approximately d 2-8 pi according to the dose, while in NDV groups shedding always started hours to days earlier, apart at the highest dose. At $10^1$-$10^3$ $EID_{50}$ the difference in NDV shedding onset was statistically significant. Analogously, analyzing the occurrence of death among challenged birds, NDV groups scored always equal or lower mean death times (MDT) compared to H9-NDV groups, apart for the $10^6$ $EID_{50}$ dose.

Shedding of H9N2 virus was assessed by measuring the mean shedding time of challenged birds and it was observed that all of the surviving birds would shed the virus from the trachea for a similar amount of time, apart from birds in group H9-NDV $10^2$ where shedding lasted approximately one day longer than the rest.

In both the experiments, when challenged birds shed the viruses, sentinels got infected, shed both challenge viruses and became sick. The only exceptions were groups H9-NDV $10^4$ and $10^6$ in which zero of the surviving sentinels neither shed the influenza virus, nor seroconverted to it.

## DISCUSSION AND CONCLUSIONS

An active H9N2 infection in SPF chickens has proved to lower the minimum infective dose of the vNDV strain from $10^2$ to $10^1$ $EID_{50}$; in fact all of the challenged birds in the H9-NDV $10^1$ group became infected with NDV and developed a mild disease as confirmed by serological data. Interestingly, the disease was more severe among the sentinels than in the directly infected birds causing 40% mortality.

Throughout the second experiment, it was noticed that the H9N2 infection postponed the onset of clinical disease and the tracheal shedding of NDV. These observations were confirmed by MDT that resulted to be either equal or higher than those in the first experiment. In spite of postponing the disease, the H9-NDV co-infection caused higher mortality among the challenged birds of all groups, apart from the $10^2$ group.

The postponed onset of shedding and clinical disease are in line with what previously demonstrated by Ge et al., (1) whose experiments in embryonated eggs proved AIV have a replicative advantage when simultaneously administered with NDV viruses. Moreover, when they inoculated NDV viruses in eggs previously infected with AIV, the advantage of influenza viruses over NDV was even stronger. Only theoretical speculations can be made about interferon gamma or receptor competition being the main mechanisms of interference between AIV and NDV.

On the other side, the increased mortality in H9-NDV groups and the capacity of the H9N2 virus to trigger the NDV infection at a lower dose, appear to relate with the existing literature on co-infections of LPAI and other pathogens (2,5). In fact, H9N2 might play a role in the exacerbation of the disease, either by affecting the immune system, or by impairing the local physical defenses at the level of the respiratory/enteric tracts.

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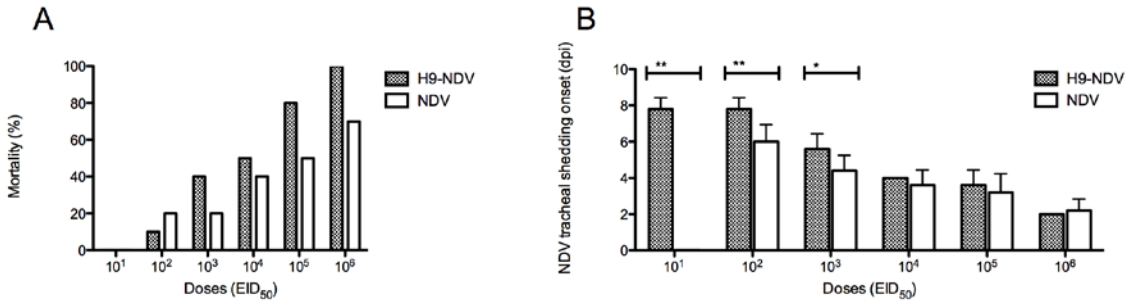
**Fig. 1**. A) Mortality of challenged birds in the two experiments; B) Mean onset ± standard deviation of NDV tracheal shedding among challenged birds in the two experiments.
**=P<0.001; *=P<0.01; dpi= day post infection



# EVALUATING ALTERNATIVES TO ANTIMICROBIALS WITH A NECROTIC ENTERITIS EXPERIMENTAL MODEL IN BROILER CHICKENS

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Increased awareness of antimicrobial resistance has seen the development of commercial alternatives and strategies to raise chickens. However, the control of necrotic enteritis (NE) remains a challenge, especially when raising antibiotic-free flocks. NE is well controlled with antibiotic feed additives, but while pathogenic strains of *Clostridium perfringens* (CP) are causal agents, they need risk factors such as coccidiosis or decreased peristaltism for the disease to happen.

Our objectives were to test the efficacy of some strategies and alternatives to control or reduce mortality during a NE experimental infection. Four sets of experiments were done, each involving four repetitions of six treatments. These consisted in the administration of:

1. Inorganic or organic water acidifiers at 2 pHs
2. Commercial essential oils (EO)
3. Organic acids, yeast or bacitracin added to the diet
4. A combination of organic acid in water and essential oils in feed.

The infection model included increased high wheat content in the diet, vaccination against coccidiosis and four consecutive oral inoculations of three field *Clostridium perfringens* strains.

None of the treatments fully prevented the occurrence of NE. Mortality and typical lesions were observed in all infected groups, even the bacitracin groups. Mortality is usually inferior to 6% with our model; however it went up to 15% in Trial 2. This increase was later explained by an accidentally higher first inoculation dose. Lack of bacitracin efficacy to prevent NE in Trials 2 and 3 was later shown to be associated to bacitracin resistance in one of the inoculated *Clostridium perfringens* strains. Cecal *Clostridium perfringens* counts showed no statistical difference between the treatments, at 0dpi and 2 dpi, for trials 1, 2 and 4. However, for Trial 3, three treatments (EO based) showed no increased *Clostridium perfringens* counts between 0 dpi and 2 dpi. Gross and histopathological lesion scorings were also lower for these treatments.

Our results show that while none of the alternative treatments prevented NE mortality, essential oil based treatments showed no increase in post-inoculation cecal *Clostridium perfringens* counts. Further investigation of this bacterial count control mechanism could prove interesting.

# SURVEY OF HEMAGGLUTINATING VIRUSES IN SPOTTED NOTHURA FROM THE PROVINCE OF BUENOS AIRES, ARGENTINA

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## INTRODUCTION

The avian influenza virus (AIV) has been isolated from a variety of animals, but the normal host is considered to be in the wild birds (8). However, in general these viruses described as Orthomyxovirus, do not cause clinical signs in free living birds. AIV can produce an array of syndromes in domestic poultry that can range from an asymptomatic infection to respiratory signs, deceased egg production, to a severe systemic infection with high mortality. The severity of clinical signs and pathology due to AIV depend on the pathogenicity of the virus weather it is a low or high pathogenic strain, the presence of concurrent infections, age, species of birds, and nutritional and environmental factors. These viruses have a worldwide distribution and a wide range of avian host spectrum. It is known that migrating water fowl and others may serve as main reservoirs. AIV has a helical symmetry containing glycoproteins. These glycoproteins project from envelope and have hemagglutinating and neuraminidase activity. They are divided into subtypes based on the antigenicity of hemagglutinin and neuraminidase. Numerous surveillance studies showed that all 16 hemagglutinin and 9 neuraminidase subtypes of influenza could be found in wild birds (4). The presence of these antigens may not correlate with virulence. However, strains with H1, H5, H7 antigens are considered pathogenic to poultry. Virulence may be influenced by interaction between strain of virus, host species and environment. It influenced also by tissue tropism (local versus generalized) and type of host protease. They have high rate of genetic recombination. Transmission takes place through respiratory secretions, conjunctiva and feces (9).

Avian influenza (AI) is one of the greatest concerns for public health that has emerged from animals in recent years. Highly pathogenic AI (HPAI) caused especially by subtype H5N1, represents one of the major animal health problems worldwide. However, no Asian-lineage HPAI viruses of the H5N1 have been detected in the Americas. Notifiable AI (NAI) infections have led to significant human health issues including the risk of generating a pandemic. In the last decade infection in humans associated with poultry outbreaks have been observed in the Netherlands with H7N7, Canada with H7N3, and in Asia and Africa with H5N1. Surveillance is an essential activity for the disease control and early detection allow the implementation of control measures. Although surveillance in backyard chickens is another way for disease control (3), a surveillance in free living wild birds in the Province of Buenos Aires and Santa Fe, started in October 2008.

Since October 2008, samples such as cloacal and tracheal swabs plus a pool of organs that have been harvested from freshly dead road kill birds or injured and fresh fecal samples, have been collected and examined. Samples were pooled according to date and area to determine whether avian hemoagglutnating viruses are present and circulating in spotted nothura (*Nothura maculosa*), previouly called spotted tinamou, (5,6) from the Province of Buenos Aires, Argentina. As a consequence of the first isolation of an avian influenza virus from the Tinamiformes order worldwide that was done from tissues belonging to clinically affected birds, a monitoring of swabs, organs pools and fecal samples from this type of birds, was evaluated. The results from October 2008 through December 2012 will be presented here.

## MATERIALS AND METHODS

**Examined birds**. *Nothura maculosa* were examined in birds. Since October 2008 samples as cloacae and tracheal swabs, as well as a series of organ and tissue samples were collected from dead or wounded birds. The samples were transported to the laboratory at 4ºC

**Transport means**. PBS pH 7.0 to 7.4 plus antibiotics or nutritive broth with antibiotics.

**Virus isolation and detection**. Over 100 samples were catalogued by date and area of recollection, each pool consisted of five samples or less. The pools were processed within four d of the recollection and kept refrigerated. Five specific-pathogen-free (SPF) chicken embryos, nine to eleven days old were inoculated via allantoic cavity, incubated and candled for five d and then refrigerated. The allantoic fluid of live and dead embryos was tested for hemagglutinating activity by a direct hemagglutination test. Blind passages were performed on negative samples to confirm the absence of hemagglutinating agents.

Once every month, two additional embryos were inoculated with the Newcastle disease (1) virus La Sota as a control for checking inoculation technique and hemagglutination tests. Allantoic fluids from live and dead embryos were tested for hemagglutination activity, and two blind passages were performed on the negative samples. Since 2006, real-time reverse-transcription PCR (RRT-PCR) for matrix (M), H5, and H7 genes using primers previously described (7) has been available at the central laboratory of Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), and this technique is available once the virus is isolated or in case the attempt of isolation cannot be accomplished and a threat of AI is suspected. A positive control virus of type A, lyophilized and inactivated, was kindly provided by Dr. I. Capua from the Istituto Zooprofilattico Sperimentale delle Venezie, World Organization for Animal Health (OIE)/Food and Agriculture Organization of the United Nations (FAO) and National Reference Laboratory for AI, Padova, Italy. Cloacal swabs from birds free from AI were used as negative controls. Briefly, 200 mL of PBS suspension from cloacal swabs were used to extract the RNA using the Total RNA Isolation Chemistry Starter Kit (Applied BiosystemsTM, Foster City, CA) in accordance with the manufacturer's instructions. RNA was eluted in a final volume of 100 mL and stored at 280º C. Viral cDNA was prepared using 30 mL of viral RNA and the High CapacityH cDNA Archive kit (Applied Biosystems) random hexamers in a final volume of 60 mL following manufacturer's directions. The cDNA was tested for AI by RRT-PCR using TaqManH Universal PCR Master Mix (Applied Biosystems), directed to the matrix (M) gene, which detects all type-A influenza viruses, as previously reported (7). The PCR reaction can be performed in an ABI Prism 7900 Sequence Detection System apparatus (Applied Biosystems).

## RESULTS AND DISCUSSION

None of the sample pools showed hemagglutinating activity therefore until now none of the samples tested positive. No AI (9) or paramixovirus (1) were isolated from the *nothura maculosa*, a non-migrating bird of the tinamiforme order. In any case the survey should continue and a greater number of samples should be examined. The possibility of adding RTq-PCR to detect specific RNA AIV should be considered. The first isolation of influenza virus in the tinamiforme order was achieved using tissues of clinically affected birds (2). Those belonged to hunting grounds related to a swine farm. The birds analyzed in this study were healthy and the circulation of these viruses could not be determined. More studies are necessary to determine the role of these birds in the epidemiology of hemagglutinating virus if there is any.

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*Note: The institution for which I worked also during the first year of the study was Cátedra de Zootecnia Especial III (Aves y Pilíferos), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina.

# INTEGRATED CONCEPT OF VACCINATION AND PHAGE THERAPY FOR CONTROL OF BACTERIAL ENTEROPATHOGENS IN FOWL

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Enteropathogenic bacteria like *E. coli*, *Salmonella*, *Campylobacter* and others in fowl, meat, or products thus represents a considerable health hazard for human. With the reduction of antibiotics in the feed there is a high likelihood that the load of fowl with these pathogens increases without a novel animal management system. The strategy underlying our concept is based on the reduction of pathogens in the environment and within the carrier by phage therapy. At the same time, the innate immune system of the animals should be stimulated by Bacterial Ghosts (BGs) derived from non- pathogenic bacteria. BGs can be produced from Gram-negative bacteria by a BIRD-C proprietary technology and are characterized as the native empty shells (cell envelope complex) of pathogenic or non-pathogenic bacteria. Production of BGs follows the following steps:

1. A plasmid carrying the PhiX174 gene E expression cassette is introduced into Gram-negative bacteria.
2. The bacteria carrying the gene E expression cassette are grown and at a defined time during the growth period of the bacterium gene E expression is activated. Protein E molecules integrate into the bacterial cell envelope complex and form a transmembrane hole by fusing the inner and outer membranes at potential division sites.
3. The cytoplasmic cell content is expelled through the E-lysis hole and the remaining bacterial envelopes (BGs) are harvested and washed.
4. Remaining DNA and non-lysed bacteria are chemically inactivated by beta-propiolactone.
5. The BG concentrate is aliquoted, stored at 4°C, frozen or freeze-dried.

BGs have several advantages compared to conventional inactivated bacterial vaccines. They are non-living and carry no pathogenic threat unlike attenuated bacteria; horizontal gene transfer of pathogenic islands to other bacteria is not possible unlike attenuated bacteria and bacteria inactivated with state-of-the-art methods. BGs the natural surface components and full immunogenic potential unlike classically inactivated bacteria; there is no requirement for the addition of adjuvants to elicit complete immune response (needed for recombinant subunit-, and classically inactivated bacteria vaccines, virosomes, and liposomes). It is possible to create multivalent vaccines targeting various pathogens at the same time (viral, bacterial, and eukaryotic). BGs are produced by rapid and cost-effective production by bacterial fermentation (no need for eukaryotic production system, as for virosomes). BGs exhibit high stability and immunogenicity of the finished product at ambient temperature for several years in freeze-dried formulation (unlike all competing technologies). BGs proved strong immunogenicity after mucosal administration (unlike virosomes/liposomes). BGs derived from pathogens are very effective at inducing a strong humoral and cellular immunity against the bacterial pathogen and often produce cross protection to related serovars (1). BGs of *Salmonella* Enteritidis have been used successful to confer protection against internal organ colonization and egg contamination of laying chickens (2).

The other intervention to control enteropathogens is to use phages to destroy bacterial pathogens in the environment and in the gut of the animals (3). Phages have been used in the past successfully as summarized in Table 1 (4). Phages have the intrinsic ability to identify their target bacteria and depending of the phage type to replicate within the host. This then will kill the targeted bacteria by lysis by release of progeny phages which are again able to infect new bacterial host cells. Appropriate virulent phages with these features can be selected from the environment (for enteric pathogens fecal samples or sewage) and tested for their killing efficiency against the bacterial pathogens in question, e.g. *Salmonella enterica* serovars. Phages are then screened for selected features; for example adsorption (binding) and binding requirements to the host candidate(s), burst size, growth at industrial culture conditions for production of high-titer stocks, stability at storage and in the animal and environment. Total genome sequence and identification of genes for toxins or antibiotic resistance are other requirements. The question of single phage versus phage cocktails largely depends on the availability of suitable phages and target host range. Still broad host range phages or cocktails display a much

narrow spectrum of activity than antibiotics and avoid undesired antibiotic associated effects. Finally, establishment of phage biocontrol protocols, costs and market acceptance will influence the novel food safety invention strategies.

In combination with the BG immunization system it is desirable to select phages which during bacterial lysis do not destroy the bacteria completely. These leave the cell envelope almost intact or in large fragments. These can easily be recognized by the professional antigen presenting cells (e.g., dendritic cells, macrophages) of the immune system by pathogen associated molecular patterns, bound antibody Fc receptors, or others to initiate either a primary immune response in the animal or to booster an BG pre-induced immunity. In this sense a phage-based biocontrol of bacterial pathogens can be achieved which is stronger than each of the two components alone.

Immunization or strengthening innate immunity of hatched chicken can start at d one providing BGs of the target pathogen or BGs are derived from non-pathogens in drinking water and can continue by adding them to the feed. Other routes of immunization which have been tested in various animal trials are subcutaneous, intramuscular, or BG delivery as aerosols. In the extended version BGs can also be carrier of additional antigens coming from other Gram-negative or Gram-positive bacteria, from viruses or parasites leading to BG combination vaccines.

Since BGs are nonliving they do not possess the hazard to revert to a pathogen or to become carriers as can be considered for life attenuated vaccines. On the other hand, these field isolates are the same BG vaccine candidates as described above. Within BG immunized animals the cell envelope fragments and cytoplasmic pathogen factors are released after phage killing can act as booster or to enlarge the antigens for soluble cytoplasmic components. BG vaccination and phage therapy go hand in hand and are potent arms to fight against undesired enteric pathogens in fowl.

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**Table 1.** Bacterial phage past applications.

| |
|---|
| Reduction of E.coli O157:H7 on pre slaughter cattle hides in Argentina |
| Spray cold cuts to eliminate Listeria monocytogenes in Scotland |
| Elimination of Listeria monocytogenes from RTE salmon |
| Chronic wounds therapy and dermal ulcers in Russia |
| U.S Afghanistan and Iraq US Army personal wounds infected with Acinetobacteriabaumanii |
| MRSA and VRSA clinical patients infected with Staphylococcus spp. in USA |
| Rats experimental infected with Staphylococcus in USA |
| Controlling poultry Salmonella by injecting incubating eggs with phages at day 17-19 |
| Prevention of a future waterborne outbreak for O157 in Ontario, Canada. |

# DIAGNOSIS OF A LOW PATH AVIAN INFLUENZA H5N8 AMERICAN STRAIN IN COMMERCIAL QUAIL LAYERS IN CENTRAL VALLEY CALIFORNIA

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## INTRODUCTION

Increased mortality was the reason for submission of 15 live and five dead 15-wk-old, Japanese quail hens (*Coturnix c. japonica*) to the California Animal Health and Food Safety Laboratory (CAHFS), Turlock branch. Mortality went from approximately 0.013% per week to over 3.0% in two weeks. Only bilateral swelling of the eyelids was noticed at clinical inspection of the birds. Postmortem examination revealed mild congestion of the lungs and multifocal foci of necrosis and hemorrhages in the liver and pancreas. Avian influenza virus (AIV) H5 was detected by rRT-PCR from oropharyngeal and cloacal swabs. Subsequently, the virus was isolated in tissue cultures and specific pathogen free embryonated eggs. Pathogenicity index was determined by analysis of the sequence of the hemagglutinin gene at the cleavage site and by *in vivo* assay in chickens. The virus was classified as influenza A/quail/California/K1400794/2014(H5N8).

Histology of pancreas revealed severe extensive coagulative necrosis and severe lymphoplasmacytic inflammation. Severe centrolobular necrotic hepatitis was also present associated with moderate mixed inflammatory reaction and mild hemorrhages. Tissue samples including lung, trachea, larynx, liver, pancreas, spleen, brain, kidneys, adrenals, intestine, proventriculus, air sacs, ovary and oviduct were analyzed by immunohistochemistry with an assay developed to study the tissue distribution of the AIV antigens. The amount of antigen present in each tissue was scored subjectively as negative, minimal, moderate, and abundant on the basis of the estimated number of positive cells. The outbreak was reported to the OIE due to the potential of low pathogenic AIV H5 to mutate to high pathogenicity. The infected operation was depopulated, cleaned and disinfected. Surveillance testing established that the virus did not spread from the index flock.

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(This article will be submitted to *Avian Diseases*.)

# PHALLUS DISEASE IN GOOSE BREEDERS: PATHOLOGICAL AND BACTERIOLOGICAL FINDINGS

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## SUMMARY

Poor fertility in a commercial goose breeder flock in California was the reason for submission of six live Toulouse ganders to the California Animal Health and Food Safety laboratory, Turlock branch. The farm had multiple breeds of ducks and geese which were raised for eggs, balut, meat, and other related products for ethnic markets in California. The Toulouse flock, which consisted of approximately 410 birds, 90 males and 320 females, was dominantly affected. During the 2014 breeding season, fertility dropped from approximately 65% in February to less than 40% in March. However, no impact on the egg production was noticed. Subsequently, inspection of the flock found 44.4% of the Toulouse ganders with phallic alterations which prevented them from mating. Necropsy of affected ganders revealed severe granulomatous inflammation disrupting the architecture of phallus. In addition, multifocal lymphoid nodules were seen in the mucosa and submucosa of the phalluses, causing constriction of the spermatic and ejaculatory ducts. The lesions observed were confined exclusively to the male genital tract. Three different species of mycoplasma were isolated from the phalluses and sent to The University of Georgia for characterization. Polymerase chain reaction protocols targeting the 16S-23S rRNA intergenic spacer (ISR) regions (1) and the RNA polymerase beta subunit (rpoB) gene (2) were performed on the isolates. Three distinct isolates were identified upon sequencing and analysis using NCBI BLAST; *Mycopasma cloacale*, *Mycoplasma anseris*, and an unknown novel *Mycoplasma* spp. In addition, *Pasteurella multocida*, in combination with other bacteria, was also isolated from the phallic lesions and identified as serotype 3 with a DNA profile of 1511 (at NVSL).

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(This article will be submitted to *Avian Diseases*.)

# OPTIMIZING INTESTINAL HEALTH IN BIRDS RAISED WITHOUT ANTIBIOTICS

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## INTRODUCTION

A 2012 survey of the US broiler industry to determine and rank production challenges indicated that gut health management was paramount in the minds of those involved with live production (23). This is not surprising since intestinal integrity determines feed efficiency, the most important economic driver of the meat industry. Since then, some significant changes in the industry have sharpened the focus on managing intestinal health.

The Poultry industry is experiencing a particularly profitable period. Over the last twelve months, a nationwide shortage of pork, beef and chicken has induced a significant strengthening in the price of meat and this has coincided with a fall in the price of corn, the primary ingredient in US animal diets (35). While this would normally ease focus on the intensity of intestinal health management, this has not been the case because the status quo of traditional bacterial and protozoal enteropathy control strategies have been concomitantly been shattered by three occurrences. Firstly, the inference that the prevalence of gangrenous dermatitis is associated with the use of ionophores (46), secondly, the voluntary removal of 3-nitro from broiler feed in 2012 and thirdly, a recent statement of intent by a leading fast food chain to only sell chicken meat raised without in-feed antimicrobials within the next five years (5).

## FROM INTESTINAL-FLORA TO HOUSE-FLORA

Although poultry meat production systems are all-in-all-out in nature, they are, from a gut flora perspective, a continuous system. Members of the gut microbial community surviving in the house environment are carried over from one cycle to the next and thus serve as the "seed stock" for the gut flora of the next placement (33). While in-feed antibiotics can alter the gut flora within a couple of weeks, it takes several grow-out cycles to change the house (litter) flora (3, 29, 33, 48, 49). This is by no means a new concept, both rotation and shuttle programs have been used for decades to avoid the lack of response to in-feed antibiotics following their persistent use.

The realization that even minor changes in intestinal microbial community composition can affect long term productivity through incremental displacement and replacement of the house flora has highlighted the significance of microbial community management (40). Attention to detail is more critical than ever. The efficiency of nutrient assimilation hinges on the early establishment and maintenance of a favorable gut lumen environment. In a drug free production system the emphasis shifts from fighting the unfavorable organisms with antibiotics to nurturing the favorable organisms; working with nature to ensure a favorable and stable intestinal ecology. In its simplest format this involves: *seeding* the gut with favorable intestinal microbiota, *feeding* these organisms to ensure that they rapidly dominate the intestinal microbiota and *weeding* out the unfavorable organisms.

## SEEDING THE GUT WITH FAVORABLE ORGANISMS

The first organisms to colonize the gut direct the evolution and composition of the climax flora by creating the microenvironment necessary for complex microbial community development (27). Colonization of the gut with pioneer bacteria species, that are able to modulate expression of genes in the gut epithelia to optimize nutrient assimilation and create favorable conditions for establishment of a stable and beneficial climax flora, should be the starting point of any gut health management program (21, 27). In addition, competitive exclusion has long been recognized as a means of preventing pathogen colonization of the intestinal tract and probiotics have recently been shown to suppress colonization of the intestine with *Brachyspira pilosicoli* (34), *Clostridium perfringens* (42), *Campylobacter jejuni* (45), and *Salmonella enteritidis* (55). Since the first organisms to gain access to the hatchling gut originate from the parent, steps to control gut health

should start at the parent flock level. Vertical transmission of gut inhabitants (from parent to offspring) can be transovarial (inside the egg) or as a result of contamination during oviposition (28, 30, 41)

In the artificially clean hatchery environment, even low doses of beneficial bacteria can significantly improve resistance to pathogen colonization, and artificial seeding of the gut at an early age has been shown to be beneficial (6, 15-17, 19, 24-26, 32, 36, 38).

## FEEDING THE FAVORABLE ORGANISMS

In addition to seeding the gut with the correct pioneer species, it is crucial to enhance their ability to proliferate, compete and colonize, so as to avoid pathogen proliferation. Weak organic acids can be used to change gut flora community structure (39, 43). As weak proton donors, they are able to escape inactivation in the upper intestinal tract (proventriculus and gizzard), while their presence in the small intestine modifies microbial community composition (44). Endogenous short chain fatty acids have a microbiota stabilizing effect and butyrate in particular has been shown to stimulate the production of host defence peptides (β-Defensins and Cathelicidins) (54). By providing a competitive advantage to the acid tolerant organisms such as the Lactobacilli and a competitive disadvantage to the acid intolerant organisms like the Clostridia, it is possible to guide the development microbiota composition (13, 44). Such manipulation of the microbiota has both short and long term implications.

Unfavorable organisms are in general much more competitive in the environment of the lower intestinal tract and their replication is normally kept in check by intense competition for a limited source of nutrients (58). Any factor that reduces digestion efficiency in the upper gastrointestinal tract, or increases nitrogen turnover in chickens, could potentially alter cecal ecology. Urine (uric acid) and feed (undigested protein) nitrogen are used by cecal flora to synthesize microbial protein (4), a process that unfortunately yields toxic metabolites and causes dysbacteriosis (12, 14, 22). In contrast, volatile fatty acids (VFA) formed during carbohydrate degradation, have antibacterial activity which has a stabilizing effect on the cecal ecology (2, 7, 8, 13, 53, 56, 57). Since cecal ecology is adversely affected by protein maldigestion, exogenous enzymes designed for protein ingredients can be used to help stabilize cecal flora communities. The amount of protein nitrogen reaching the ceca can be further reduced if nutrient credit allocation permits a reduction in dietary protein (47).

## WEEDING OUT THE UNFAVORABLE ORGANISMS

The traditional approach to weeding out unfavorable organisms has been through the addition of a low level of antibiotic to the diet. The consumer has, rightly or wrongly, made the link between the emergence of antibiotic resistant strains of human pathogens and antibiotic use in animal agriculture. This approach to intestinal microbiota management is rapidly falling from grace. While antimicrobial substitutes such as essential oils and in-feed bacillus probiotics have become popular, the long term sustainability/future of these products may come into question; they are after all antibiotics by a different name. Alternatives that utilize a different mechanism of action which avoids the negative aspects of low dose antimicrobial use is, from most perspectives, a more suitable solution.

As colonization proceeds, organisms attach to one another and the epithelium by a series of fibrils, to form a tightly adherent mat over the gut surface (20). Pathogens are thereby precluded access to the epithelial surface and their ability to colonize is compromised by a process of competitive exclusion (37). Microbe attachment to host cell docking sites on the intestinal epithelium is dependent on surface molecule structure and is the pivotal first step in the colonisation and infection of the gut (20, 31, 50, 52) . Since several gut pathogens recognise and attach to specific gut epithelia glycoproteins, products that mimic these docking sites are also useful in preventing attachment and reducing the risk of pathogen colonization (1, 18, 20, 51).

Pathogen induced inflammation of the gut lining stimulates mucus secretion, increased paracellular permeability, and accelerated feed passage (peristalsis) (10, 11). The cascade of events that follows is self-perpetuating. Increased permeability enhances toxin and agent penetration, which in turn stimulates inflammation, and the resulting increase in mucus production attracts mucolytic species such as *Clostridium perfringens*, which produce tissue damaging cytotoxins (9, 10); a vicious cycle ensues.

## CONCLUSION

Strategies to improve gut health in commercial operations need to be cost effective, sustainable, farm specific and holistic. Intervention / product selection needs to be science based but practical and each intervention must address the specific objective for its inclusion. Efforts to nurture and stabilize a favorable intestinal microbiota with alternative approaches have shown promise in addressing the

negative impact of in-feed antibiotic removal and use. While there are several opportunities and product options to achieve this, there are three simple interventions that have demonstrated particular promise. By *seeding* the hatchling gut with favorable organisms, *feeding* these organisms with an appropriate organic acid, and *weeding* out the unfavorable competitors with a type-1 fimbriae blocker, it is possible to improve performance by accelerating the evolution of, and maintain the stability of, a favorable intestinal microbiota.

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# A COMPARISON OF VACCINATION PROGRAMS AGAINST A CONTEMPORARY CAL-99 IBV ISOLATE IN COMMERCIAL BROILERS

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## INTRODUCTION

Infectious bronchitis is one of the top economically costly broiler diseases in the United States, regardless of geographic region. A California variant, Cal-99, continues to circulate and trigger respiratory disease in California broilers. Over ten years ago, a challenge study (1) showed that Mass + Ark gave more complete protection than Mass + Conn. Other research showed that while Ark-DPI has the highest percent similarity to Cal-99 viruses in the S1 region (3), Cal-99 IBVs are nonetheless a distinct serotype (2). The goal of this study was to compare protection levels afforded by several different vaccine combinations, with or without a booster, against a contemporary Cal-99 field virus.

## MATERIALS AND METHODS

A total of 120 commercial newly hatched broiler chicks were equally divided into eight different treatment groups. For each treatment, birds were placed into either two or three Horsfall isolator units, depending on isolator capacity (5 or 10 birds). Treatments 1 and 2 were not vaccinated and served as negative and positive (Cal-99 challenged) controls, respectively. Treatments 3-8 were all challenged via eye drop with 4.5 EID50(log10) Cal-99 contemporary isolate K1400827-01.0018 at 31 d after first being vaccinated via eye drop at hatch +/- a 17 day booster. Vaccine treatments were as follows:

3) Mass+Conn
4) Mass+Ark (DPI strain in all)
5) Mass+Ark → Mass+Ark
6) Mass+Ark → Holland+Ark,
7) Mass+Ark+Ga98
8) Holland+Ark.

The effect of the various vaccination treatments was evaluated and compared at five d post-challenge (36 d of age) using the following parameters:

- Clinical Signs: 0=None; 1=mild respiratory noise; 2=constant noise, rasping; 3=respiratory rales.
- Airsacculitis: 0=None; 1=mild suds; 2=moderate suds or exudate; 3=heavy suds or profuse exudate.
- Histopathology: 1=no inflammatory cells; 2=inflammation, hyperplasia, deciliation, increased goblet cells, mucous; 3=any type of necrosis; 4=any amount of ulceration.
- Real-time PCR: estimation of viral loads using Ct values (# PCR cycles to get a positive signal).

## RESULTS

The Cal-99 challenged controls had the highest incidence of moderate to severe clinical signs (47%), moderate to severe airsacculitis (27%) and tracheal lesions above baseline (73%). The challenge controls also had the highest viral loads (lowest mean Ct value), indicating a successful Cal-99 “take”. Protection from moderate to severe clinical signs seemed to be the easiest standard to reach as all vaccine treatments were 100% protected. The incidence of tracheal histopathology in challenge controls (73%) was much higher than all other groups (0-13%). Airsacculitis scores seemed to show the least separation between vaccinated and non-vaccinated challenged groups (0-13% vs. 27%, respectively), although the two-time Mass + Ark vaccination group was the only group to have none. Ct values seemed to fall in five general ranges, with the negative and positive controls framing the high and low end (40 and 21.3, respectively). The one-time Mass + Conn vaccine group was only about four cycles higher than the challenge controls for the lowest viral reduction of any vaccine intervention. The two-time Mass + Ark group gave a Ct value about 10 cycles higher than the challenge controls for the highest viral load reduction. The other four vaccination programs gave similar mean Ct values between the two aforementioned vaccine treatments (seven to eight cycles above Cal-99 controls).

## DISCUSSION

The incidence of moderate to severe clinical signs and trachea lesions was highest in challenge controls but these parameters could not differentiate between vaccine treatments. Airsacculitis scoring was even less discriminatory, although the 2-time Mass + Ark vaccination was the only treatment with no airsacculitis. Real-time PCR analysis, however, appeared to be the most discriminating measurement to separate different vaccination regimens. To the point, Mass + Conn reduced viral loads the least—slightly more than a single log (base 10). Mass + Ark, on the other hand, lowered viral loads by about an additional log. Similar reductions were seen with Mass + Ark + Ga98 and Holland + Ark. These results demonstrate the value of adding Ark instead of Conn to Mass in order to reduce Cal-99 infection. However, adding a third serotype, Ga98, did not enhance Mass + Ark protection, nor did the substitution of Holland for the milder Mass vaccine—perhaps because Ark vaccine was the primary driver of Cal-99 specific immunity. Finally, following up the day of age Mass + Ark with the same booster gave the greatest reduction in viral loads, suggesting that boosting programs may provide better protection in the face of a high field challenge.

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**Table 1.** Summary of all measured parameters five days after Cal-99 IBV challenge.

| | Treatment descriptions | | | Incidence or mean value of key indicators | | | |
|---|---|---|---|---|---|---|---|
| Grp # | Day of hatch Vaccination | 17 day vaccination | 31 day challenge | Percent Clinical signs >1 | Percent Airsac scores >1 | % Trachea histopath scores >2 | PCR Mean Ct Value |
| 1 | NO | NO | NO | 0 | 0 | 0 | 40* a |
| 2 | NO | NO | Cal-99 | 47 | 27 | 73 | 21.3e** |
| 3 | Mass + Conn | NO | Cal-99 | 0 | 13 | 13 | 25.4d |
| 4 | Mass + Ark | NO | Cal-99 | 0 | 7 | 0 | 28.8c |
| 5 | Mass + Ark | Mass + Ark | Cal-99 | 0 | 0 | 7 | 31.2b |
| 6 | Mass + Ark | Holland + Ark | Cal-99 | 0 | 13 | 0 | 29.2c |
| 7 | Mass + Ark + Ga98 | NO | Cal-99 | 0 | 13 | 0 | 28.9c |
| 8 | Holland + Ark | NO | Cal-99 | 0 | 6 | 13 | 28.7c |

*Assigned value when samples are considered IBV negative.
**Different letters indicate significant differences ($p \leq 0.05$) based on investigator's statistical analysis.

# HEPATITIS E VIRUS INFECTION IN ORGANIC LAYERS

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## SUMMARY

Avian hepatitis-E-virus (HEV) is a member of the genus *Hepevirus*. The clinical disease caused by HEV is referred to as big liver and spleen (BLS) disease in Australia (8) and as hepatitis-splenomegaly (HS) syndrome in North America (4). The disease has been described in broiler breeders and table-egg layers. Infections may be subclinical or associated with low mortality and mild decreases in egg production. Typically, dead birds are in good body condition. Affected birds have enlarged livers, enlarged spleens, and have subcapsular hemorrhages or hematomas. Typical histologic changes include massive coagulative necrosis, vasculitis, hemorrhage, amyloid deposition and non-specific hepatitis with a wide distribution through out the liver (1, 7). This presentation describes atypical field outbreaks of HEV in organic flocks.

## INTRODUCTION

Between 2012 and 2014, ten commercial organic flocks submitted with a history of drop in egg production (up to 40%) and slight increased mortality (up to 1%). A total of 141 birds were examined. At necropsy the most common finding was pinpoint white to tan foci on the livers (50.4%) and regressed ova (43.3%). Mild to moderate splenomegaly was observed occasionally (9.9%). Hemorrhage or accumulation of sero-sanguinous fluid in the abdomen was found only in 2.8% of the birds. Histologically, there was multifocal acute necrosis of the liver with little inflammatory reaction. No significant phlebitis or amyloidosis was observed. No parasites were detected. No significant bacteria were detected either. Although, the pathologic changes these cases were similar to reports of "spotty liver disease" or avian vibrionic hepatitis (3, 5), silver stain was negative for vibrio-like organism. Furthermore, six livers from two different submissions were tested for the presence of *Campylobacter*. *Campylobacter* was detected by PCR, but not culture, from only one liver.

HEV was detected in the livers by nested reverse transcriptase polymerase chain reaction (PCR) reaction (2). Sequencing results showed that the virus belonged to the genotype 2. Additionally affected flocks were seropositive to HEV by enzyme-linked immunosorbent assay (ELISA).

Conventional caged layer flocks housed adjacent to affected flocks never developed the disease. Because the virus is primarily transmitted via the fecal-oral route, it is possible that birds in conventional systems had no contact with fecal material and were not infected. A conventional caged layer flock flocks housed next to an affected flock tested negative for HEV by ELISA.

The clinical and pathologic presentation of HEV in these organic flocks was different from the described in the literature (6). In these cases, the drop in egg production was more severe than expected. Also, fewer than 10% of the birds had enlarged spleen, and none had enlarged liver. On the other hand the vast majority of the birds had multifocal necrotic foci through out the liver, without phlebitis or amyloidosis. Hemorrhage or accumulation of serous-sanguineous fluid was rarely observed. It is possible that this condition is under diagnosed in organic and cage free layers, as the clinical presentation may be different.

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

# THE GROWING IMPORTANCE OF NIGHTTIME AIR QUALITY

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During cool weather, maintaining good air quality is vital to avoid bird health issues. It is a 24-hour-a-day, seven-day-a-week, job. One of the most important air quality variables that must be kept to a minimum is ammonia. High ammonia levels can paralyze and/or damage a bird's tracheal cilia, which are a vital tool a bird uses to fight off disease. The cilia are microscopic finger-like projections that cover a bird's trachea. A thin layer of sticky mucus covers the cilia which can trap pathogens traveling on small particles in the air. The cilia move in a wave-like pattern pushing the pathogen-carrying mucus up to the birds' mouths where they then swallow it and the pathogens are killed off by digestive acids. But, if the cilia are paralyzed and/or damaged by high ammonia levels the pathogens can then travel through the stationary mucus layer down to the tracheal base cells where they start multiplying causing the birds to get sick (i.e. bronchitis). Worse yet, the pathogens can kill off the cilia cells which makes it easy for a wide variety of other pathogens to be introduced into the bird, leading to secondary infections (i.e. E. coli). Though long periods of high ammonia are of greatest concern, it is important to realize that research has shown that acute spikes in ammonia are potentially more damaging than constant low levels of ammonia. This is why producers have to make sure that ammonia levels are kept to a minimum not only during the day, but at night as well.

During the cooler times of the year air quality during brooding tends to be fairly consistent over the course of the day. This is because air exchange rates are determined by minimum ventilation fan timer settings which most producers leave the same 24 hours a day. Furthermore, since the young birds are not producing a significant amount of heat and outside temperatures are well below target temperatures the house never goes into "cooling" mode where ventilation rates would increase. As a result, if the ammonia levels are low in the afternoon it is typically safe to assume that they would be low in the middle of the night (Figure 1). Unfortunately, the same cannot be said for later on in the flock.

With older birds, the amount of fresh air that is brought into the house during cold weather is not solely determined by minimum ventilation fan timer settings like it is during brooding. The older the birds get, the greater the amount of heat they produce, the lower the house set temperatures, the more likely fans will operate during the day as outside temperatures become closer to inside temperatures in order to maintain the proper house temperature. As a result, daytime ventilation rates can often be two to three times as high as they are at night. Since ventilation rates tend to be higher during the day than at night, air quality can be significantly better during the day than at night (Figure 2).

Diurnal variations in air quality in poultry houses can prove problematic for poultry house managers. For the most part, air quality is evaluated during the daylight hours when the birds are being tended to. If the ammonia levels appear acceptable, ventilation rates are typically not changed. Herein lies the problem. Just because ammonia levels are acceptable during the day doesn't necessarily mean that there aren't potentially harmful levels at night. It is important to keep in mind that ammonia concentrations in a poultry house are generally proportional to ventilation rates. As a result, if ventilation rates are reduced by 50% at night because the minimum ventilation fans are only operating off of interval timers and not temperature, the ammonia concentrations will be twice as high at night then they were during the day. If the fans are only operating a third as much at night, ammonia concentrations can be three times as high at night then they were during the day (Figure 3).

The fact that air quality can vary dramatically from day to night can lead to unexplained bird health issues. It's a frustrating situation. House temperatures are correct, ammonia levels are less than 20 PPM, the relative humidity and dust concentrations are low; everything appears ideal, but mortality is climbing and the birds are starting to show signs of the onset of respiratory disease. Why are the birds becoming ill?

It is important to realize that there is always some type of disease challenge present in a poultry house. Twenty four hours a day, seven days a week, 52 weeks a year, the birds are constantly being challenged. It's not that different from the respiratory disease challenges (i.e., colds and the flu) we face during cold weather. Most of the time the bird's immune system can deal with the challenge. But, the magnitude of the challenge tends to increase during cold weather because the concentration of any pathogen that may be present in the house increase as ventilation rates decrease (for us as well as we spend

more time indoors). At night the situation can become critical as outside temperatures drop and the air exchange rates decrease to their lowest levels while pathogen concentrations and ammonia concentrations are at their highest. The combination of increased pathogen and high ammonia concentrations tend to suppress a bird's immune system. When this occurs for eight hours a night for multiple nights in a row, that is often all it takes for a respiratory disease to take hold.

So how do we know if we are ventilating enough at night to control ammonia? Ideally ammonia concentrations, like house temperatures, would be monitored 24 hours a day, but this is simply not practical. An accurate ammonia meter like the one used to produce the graphs in this newsletter costs approximately $10,000 and costs thousands of dollars a year to maintain. A second option of course would be to get up every night and check the birds at 3 am...again, not very practical. The fact is we can get a pretty good idea of what is happening at night by simply monitoring house humidity levels. Though relative humidity is primarily a measure of house moisture levels it is also an indicator of other important air quality variables such as ammonia and carbon dioxide. A high relative humidity is typically an indicator of low air exchange rates and as a result ammonia and carbon dioxide levels will also tend to be high. If there are wide variations in relative humidity levels from day to night then there are very likely wide variations in ammonia concentrations. Furthermore, if the relative humidity at night is 70 %+ on a regular basis, chances are ammonia levels are becoming excessive at night (Figure 4).

One of the best ways to monitor relative humidity is through a house's environmental controller. A modern controller can continuously monitor relative humidity and keep a record of how high the relative humidity is each night. Some controllers will even increase minimum ventilation fan runtime a set amount if relative humidity climbs above a set level which can prove extremely helpful for making sure proper conditions are maintained in the middle of the night. Another option would be to use a temperature/relative humidity data logger. Some temperature/Rh loggers are cable of recording temperature and relative humidity every five minutes for an entire flock (30 days using a one-minute sample rate) and unlike many other data loggers, displays the current readings. High/low relative humidity limits can be set and if reached they can be displayed and reset with a simple push of a button on the top of the logger.

A relative humidity sensor for an environmental controller or accurate temperature data logger will typically cost between $200 and $300. Though this may seem excessive, the information they can provide about air quality in poultry houses in the middle of the night is incredibly valuable. In the end we can do a great job of providing the ideal environment for our birds during the day but if our birds become sick because we are ignorant of what is happening in our houses at night, the cost can easily climb into the thousands of dollars for both the poultry producer and the poultry company.

**Figure 1**. Broiler house air temperature and ammonia concentrations (Day 1 - 10).

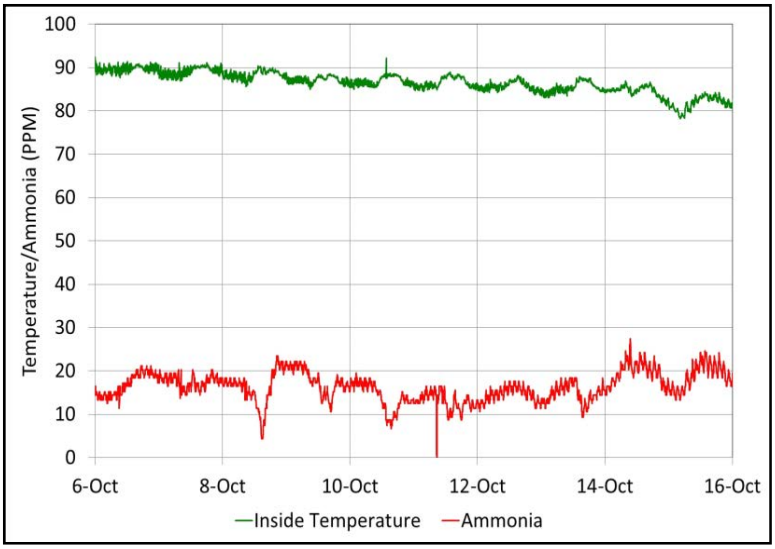



**Figure 2**. Inside/outside temperature and ammonia for a house with three-week-old birds.

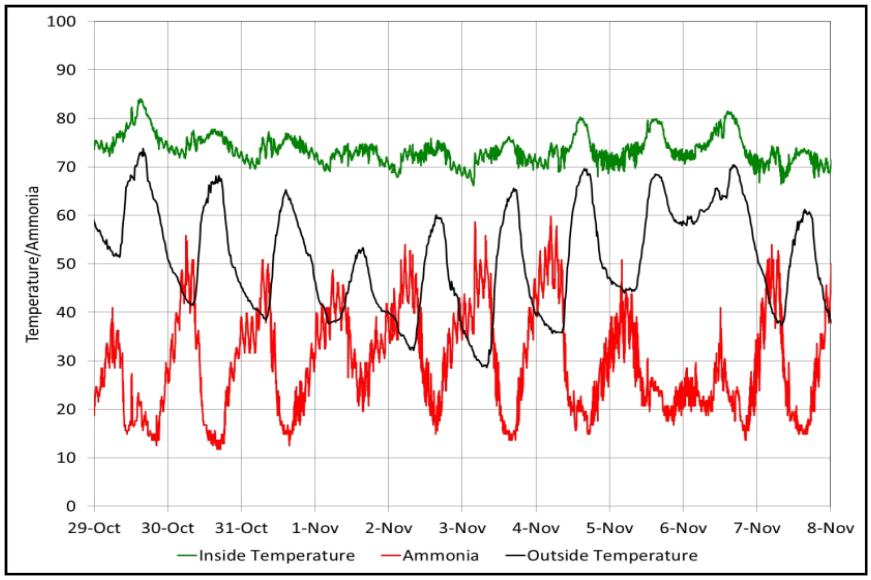


**Figure 3**. Inside/outside temperature, ammonia, and relative humidity for a house with three-week-old birds (November 3 - 4).

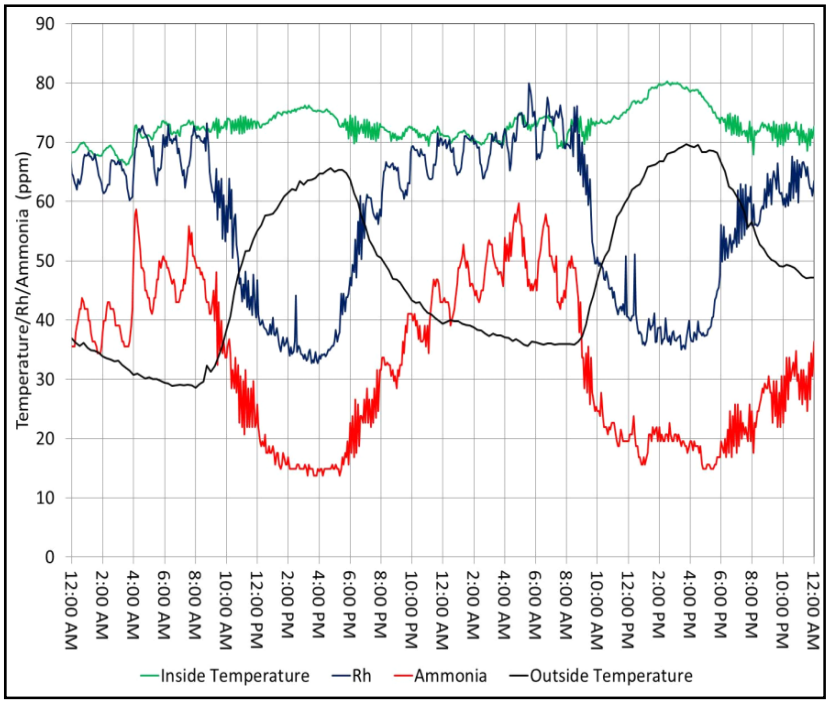
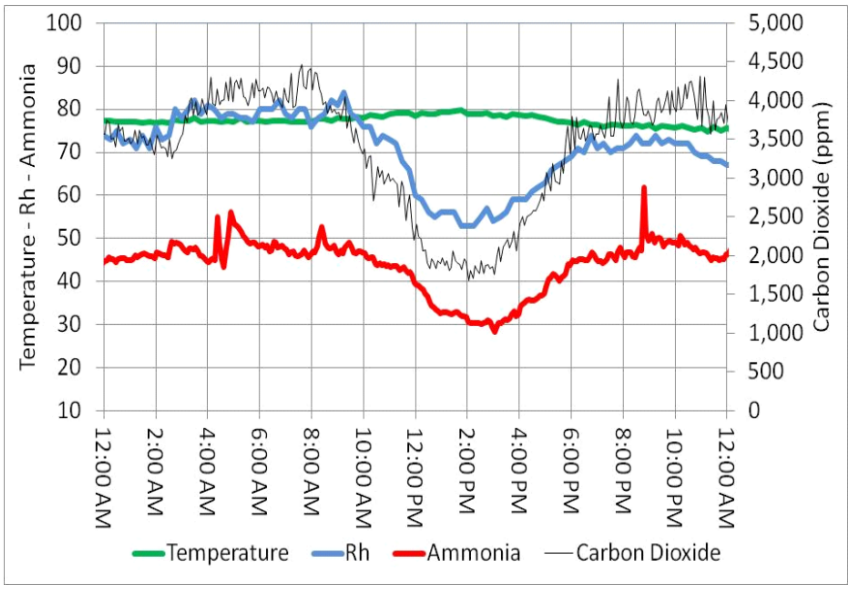


**Figure 4**. Temperature, relative humidity, carbon dioxide and ammonia levels in a house with 21 day old birds.



# ANTIMICROBIAL USE REDUCTION FOR ANIMAL PRODUCTION: OPPORTUNITY OR LIABILITY FOR THE POULTRY INDUSTRY?

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## INTRODUCTION

A nice overview of the problem of antimicrobial use in animal production is given on the EFFORT website, a European initiative for reducing antimicrobial resistance:

The introduction of antimicrobial agents in the 1940s for human clinical medicine but also in animal husbandry changed the options for treatment completely. Successful treatment of infections which were previously considered lethal became possible. In veterinary medicine, the use of antimicrobials has improved animal health, welfare and the efficiency of production.

However, the benefits of antimicrobial discovery were soon hampered as even before the moment penicillin was introduced, resistant strains of bacteria had been detected. The use of antimicrobials invariably leads to selection of bacteria that are resistant against the substance used. Resistance can then spread in populations and the environment.

In human medicine, antimicrobial resistance (AMR) leads to increased morbidity and mortality. The European Centre for Disease Prevention and Control (ECDC) estimates that AMR results annually in 25,000 deaths and related costs of over 1.5 billion € in healthcare expenses and productivity losses due to work absenteeism. In the USA, an estimated 23,000 deaths and two million illnesses were conservatively estimated by the US Centers for Disease Control.

Also in veterinary medicine the efficacy of antimicrobial treatments is declining and from several bacterial diseases (e.g. swine dysenteria) multidrug resistant strains are circulating making, these infections untreatable resulting in a high economic burden for the producers and animal welfare problems. Due to overuse of certain classes antimicrobials (e.g. tetracyclines) and beneficial pharmacological characteristics an undesirable shift towards new drugs is observed, which should preferably be reserved for human use only.

Part of the problem of treatment failure is amplified by the fact that new and effective antimicrobials are not currently being developed at a sufficient rate nor will they be developed at a higher rate in the near future. Antimicrobial resistance therefore poses a major threat to the continued efficacy of antimicrobial agents in both human and veterinary medicine.

As described above, spread of resistant microorganisms in the food chain can potentially have dramatic consequences. From emergence of resistance and along the transmission pathways, there are knowledge gaps that need to be addressed to allow for the development of science-based measures. The ultimate aim of these measures is to decrease, or at least, minimise the further development and spread of antimicrobial resistance (1,3).

## OPPORTUNITIES IN ANTIMICROBIAL USE REDUCTION

This message of antimicrobial use pressure might not be completely adopted yet by the agricultural and poultry industries, but fighting these tendencies is a losing battle. Some will consider pressure on use as a serious risk for health, welfare and especially profitability of the poultry industry, but others will look at the challenge as an opportunity.

**Respiratory and systemic infections**. Before the ban on antimicrobial growth promoters (AGP) in some countries and in most countries still using AGP, respiratory (*E. coli*, *Mycoplasma*) and systemic infections (*E. coli, S. aureus*) were and are the most common indications for antimicrobial usage. Still there is a decreasing trend of use, as control strategies include increasingly effective vaccinations against *E. coli* and *Ornithobacterium rhinotracheale* (ORT). As often diseases are multifactorial, these vaccinations must include also those targeting respiratory viral pathogens such as Newcastle disease virus (NDV) and infectious bronchitis virus (IBV). Attempts for better control of emerging diseases such as infections with *Enterococcus* spp. seem promising.

One of the main difficulties though, and thus also one of the main indications of use remaining in this area, are *Mycoplasma* spp. infections. Immunity

against *Mycoplasma* infections is not well understood; although some recent experiences with live vaccines are giving hope to design more solid programs where antimicrobial use can be optimised.

**Intestinal issues**. The main problem for the poultry industry will reside in finding antibiotic-free or low-use-antibiotic answers to gut health issues. Without AGP, about 70-80% of therapeutic antimicrobials are used for gut health issues, generally called dysbacteriosis, clostridiosis or bacterial enteritis (BE), all of these names referring to the same syndrome, globally affecting the poultry industry. As with several respiratory/systemic infections, these issues are multifactorial. At the start of the Bacterial Enteritis Vicious Circle (BEVC), a relative oversupply of nutrients in the gut creates an environment in the lumen of the gut that will help some members of the gut microbiota to abundantly replicate in disfavour of some other groups. In creating this oversupply, next to the genetic predilection of meat-type birds to high feed intakes, mostly coccidiosis is involved, and often mycotoxins, enteric viruses or other gut stressors will allow the intestinal microbiota to have contact with the gut associated lymphoid tissue (GALT), further exacerbating intestinal pathophysiological changes such as villous fusion, goblet cell proliferation, tight junction alterations, excessive GALT activation, decrease of gut peristaltic and anti-peristaltic capacities.

These changes will further, through a number of pathways, increase the availability of poorly absorbable nutrients to some bacterial groups. The result will be increased losses through poor utilization of feed and loss of nutrients due to overcoming morphological changes as well as activation of the GALT, which is the most important part of the immune system of a bird in terms of relative size of immune cells involved.

Before the ban of AGP, we were not so much aware of this BEVC creating potential losses in poultry. AGP will work to reduce fuelling the BEVC at the second step by reducing the number of potential harmful microbiota and maybe also by altering the immune reactions at the third step (4).

Understanding this vicious circle is crucial to understand the potential that some of the alternatives proposed to replace AGP have. In fact, only focusing on reducing microbial proliferation in the second step of BEVC seems to be not fully satisfying as need for retreatments often occur. This seems logical in the sense that not taking away underlying causes of initiating BEVC during the first step will of course lead to relapses. In presence of AGP this does not happen as they are given continuously, although here the use of AGP seems to cover up a constant irritation of the gut, and today it seems that better solutions can be provided by not only trying to 'replace' the AGP antimicrobial action but also looking on how to tackle the issues in other steps of the cycle. This seems in line even with the fact that also AGP results were not fully explained by the antimicrobial action (often expressed as activity against *Clostridium perfringens*) only. This understanding leads to solutions, with less antibiotics but with improved end results both in terms of welfare, health and very importantly, technical performance of poultry produced on industrial scale. This is clearly an opportunity for the poultry industry, not only in areas with ban of AGP but also when AGP are used.

## CONCLUSION

Pressure on antimicrobial use seems to be a challenge, but has already led to better understanding why and when antimicrobials are used. In the end, reduction of use will be unavoidable, but will not lead to production problems, on the contrary, it is expected that, especially in gut health, the improvements that will be made to prevent use of antibiotics will lead to performance that better approaches the genetic potential the modern meat-type birds present.

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**Figure 1.** Model of resistance emergence and the crucial roles of antibiotic and infection control in preventing outbreaks, epidemics and pandemics (EFFORT, 2014).

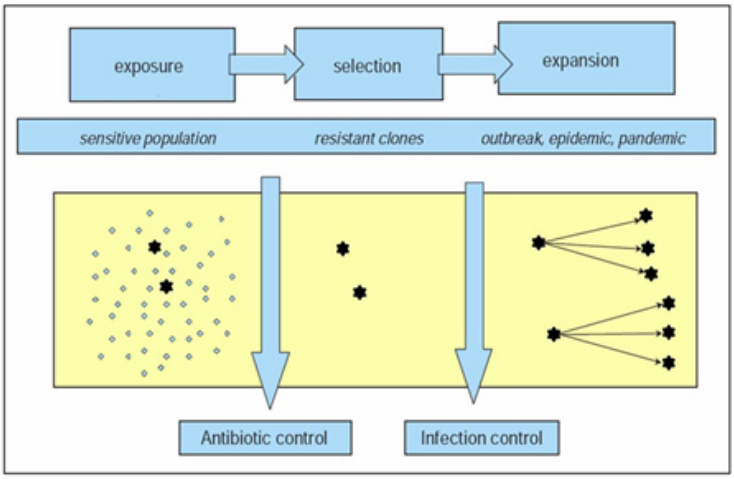
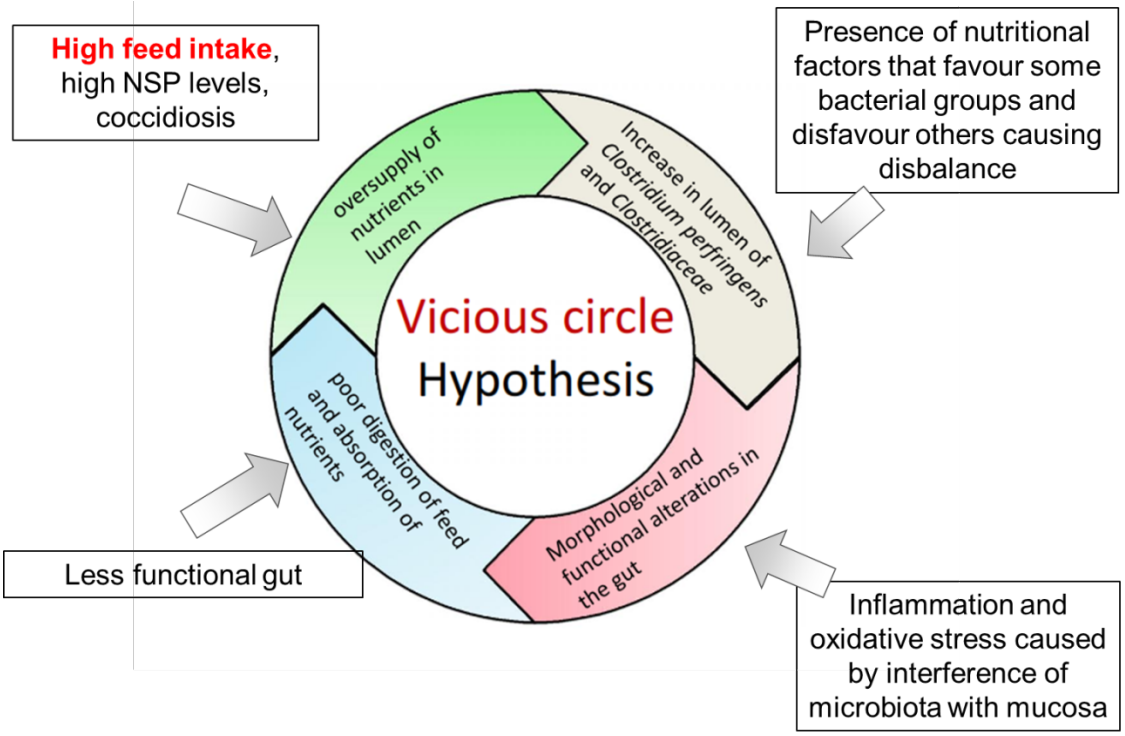



**Figure 2.** Bacterial Enteritis Vicious Circle (De Gussem, 2010).



# ANTIBIOTIC REDUCTION AND REMOVAL IN COMMERCIAL POULTRY PRODUCTION

D. Detzler

Hucon Inc./Jefo Nutrition

## INTRODUCTION

Commercial food animal production has long since relied on the sub therapeutic use of antibiotics to promote health, thus aiding in efficient, profitable production. However, actions like the 2006 EU ban on antibiotics as growth promoters (4) and the 2013 FDA announcement to phase out medically important antimicrobials in food animals for food production purposes (10) has the potential to significantly change the landscape we've grown accustomed to over the past 30 plus years.

In 2004, Hucon Poultry Inc. started to use coccidiosis vaccines to reestablish sensitivity to some of the anticoccidials that had lost efficacy (7, 2). Continuous vaccination, along with effective, more natural alternatives led to total withdrawal of all in feed and water medications in 2010. Success of this program was accomplished through changes in nutrition and management.

## WHY WE CHANGED OUR BUSINESS

With the attitude of the North American consumer, increasing resistance issues (9), and few replacement antimicrobials in development, we realized that we needed to change our production model. Deviating from the constant antibiotic regime and including such products as coccidiosis vaccines and non antibiotic alternatives allowed us to rest the antimicrobial, while reseeding our facilities with sensitive strains of Eimeria (6). If successful, this would allow us to reduce our dependence on antibiotics as well as extending the life span of such products used in our feeding program.

## ON FARM RISKS

Was antibiotic reduction or antibiotic free a reasonable goal? We were aware of small flock successes, but on the other hand, we know of sizeable negative impacts on performance and health when tried on a larger, commercial scale. Primarily, this was related to the general well being of the animal, with respect to increased disease prevalence. With the insult of challenges normally kept in check by antibiotics, performance and financials would potentially suffer. It was entirely possible that we would end up using more antibiotics to treat than to prevent.

It was also probable that due to low disease challenge in our facilities, we would be successful initially. However, it was conceivable that in the absence of antimicrobials, we could allow disease pressure to build over consecutive flocks. If the point of clinical disease was reached while producing antibiotic free in our facilities, would we be able to restore the environment back to a stable, hospitable micro flora, which we knew was favorable for bird health?

## EXPERIENCES

In the spring of 2004, we rotated from the standard regime of rotational anticoccidial use to a cocci vaccine for three consecutive flocks. Some management and nutritional changes were to be implemented so as to have the optimum chance for success. The first flock achieved fair performance. There was an increase of four points in average feed conversion (which was partially attributed to heat stress - marketing June 1- July 20). During the two following growouts, no negative health impacts were noted. Our conclusions were that a hatchery administered day of age cocci vaccine was a viable alternative to ionophores or chemicals to control coccidiosis on our farms. Upon returning to a chemical in feed anticoccidial, we observed a performance decrease in average daily gain and feed conversion. Previous commercial experience in the USA had shown the ability to reseed the poultry house with sensitive strains of coccidiosis after three flocks (1). Consequently, in our regulated "clean" environment, we were not able to reestablish sensitivity in the same time frame. Essentially, we were eradicating all we were trying to seed after the flock had been shipped. We removed litter, we cleaned, as well as washed and disinfected.

The following year, we implemented the same three cycle program. We observed a small decrease in days to market and feed conversion with each successive flock of vaccination. We questioned the ability to vaccinate through the winter flocks due to the reduced amount of ventilation during this period. A decision was made to continue vaccinating, for

cocci, but would be monitored on a flock by flock basis.

No problems with respect to disease or performance were observed. The performance continued to marginally increase, cresting around one year of consecutive vaccination.

We then focused our efforts on the chick's first seven days of life. Research has shown that early post hatch management and nutrition is essential to having a robust bird through to market (5, 8). We looked at bird migration in the barn, the needs of supplemental feed and water, lighting and temperatures. We also felt the nutritional components of our starter phase were grossly underestimated. We formulated a prestarter to emulate the swine industry's experience in the benefits of enriched early wean feeding. Early gut development was crucial. The importance of having a solid feed stimulus (birds consuming feed as early as possible) was crucial. The residual yolk contains valuable biomolecules such as maternal antibodies that are better used for passive immunity than as a source of amino acids (3).

To find out where we were exactly, we needed to establish a baseline of performance. Automatic weigh scales were installed. The data was graphed and compared to the daily growth potential published by the bird's genetic company. As a result of the data obtained, we were able to make adjustments based on certain "stalls" that were seen in the growth curve. These stalls could be correlated to the absence of proper management. With changes in place, our d seven weight increased significantly, with some flocks attaining more than five times gain from placement weight. The early gain achieved was linearly translated into end weight. Our weights were now encroaching on genetic potential. At this point, we were confident in our bird's ability to be more vigorous in defending against insults related to bacterial challenges due to further antibiotic removal.

In 2008, we started to collect, count and graph oocysts per gram of litter in our facilities. Over time, this benchmarking allowed us to establish a defined target for cocci cycling, and through specific management techniques, we were able to produce a more uniform, predictable response to cocci vaccination. With predictable cocci cycle in our houses, we started to look for opportunities to reduce the use of the necrotic prevention drug from our feed ration. Non antibiotic alternatives were tested and placed into the ration at specific times where it was felt the gram positive targeting necrotic prevention antimicrobial was not as critical. With the most efficacious alternative, we were able to remove the necrotic prevention antimicrobial in the starter and finisher rations without sacrificing zootechnical performance. The only antimicrobial used in our rations was in the grower feeds, which was from d 14 to 28.

Experience and constant benchmarking allowed us to refine our management and nutrition, taking us further into development of an antibiotic free regime. Performance to date is acceptable with an expectation of 1 to 2% in increased mortality and 2 to 6 points higher feed conversion.

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# JEJUNAL LENGTH AND WEIGHT IS INCREASED IN COMMERCIALLY RAISED TURKEYS WITH DEPRESSED GROWTH: GROSS MORPHOMETRICS

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## SUMMARY

Light hen turkey flocks raised at NCSU, CVM Teaching Animal Unit (TAU) have historically reached their target weight for processing approximately two wk earlier than sibling flocks raised on commercial farms. Comparison flocks have had the same breeder source, genetic lines, and nutrition but differed in management/husbandry practices. Unpublished data from previous studies suggests growth differences were related to intestinal health and morphology.

This study evaluated gross jejunal morphometrics between two turkey sister flocks (A and B) raised under different management conditions. Both flocks were followed from day of hatch through processing of flock A, which reached target weight first. Both groups were fed the same diet *ad libitum*. Each flock was comprised of two genetic lines of turkey hens in equal proportions that were commingled. Genetic lines were differentiated via toe trim pattern. Poults came from the same breeder flocks and were hatched together. Ten clinically normal birds from each genetic line in both flocks were sampled weekly (n = 40 samples/wk). Individual body weights, jejunal weight, and jejunal length were measured and samples taken from the intestinal tract and placed into 10% neutral buffered formalin for future study. Relative jejunal weight (jejunal weight/body weight X 100), jejunal density (jejunal weight (g)/jejunal length (cm)), and jejunal efficacy (body weight (g)/ jejunal length (cm)) were calculated. Additionally, Ussing chamber analysis was conducted in birds from one genetic line in both flocks at wk three to assess intestinal barrier function, which measured movement of mannitol across the intestinal mucosa.

Flock B experienced depressed growth and took 13 d longer to reach target weight compared to flock A. After wk one, a significant increase in jejunal length occurred in flock B turkeys compared to flock A turkeys even though flock B turkeys were lighter weight. This relative increase in intestinal length remained consistent throughout the growout period. Furthermore, jejuna of flock B turkeys were significantly heavier than those of turkeys in flock A. Within both flocks there was no consistently significant difference between genetic lines comprising the flocks. Relative jejunal weight and jejunal linear density were increased significantly in flock B turkeys while jejunal efficacy was significantly increased in flock A turkeys. Turkeys in flock A, irrespective of genetic line, were able to support more body weight per centimeter of intestine than turkeys in flock B. Flock A birds reached target weights more quickly than their counterparts in flock B even though they had longer, heavier intestines.

Ussing chamber analysis between turkeys of a single genetic line in both flocks A and B showed increased transepithelial resistance (TER) and unidirectional flux ($J_{ms}$) of birds in flock B, which indicates intestinal injury and impaired function. Functional deficiency coupled with increased jejunal mass is consistent with compensatory hypertrophy and/or hyperplasia to make up for reduced intestinal function.

Results of this study suggest that as intestinal function becomes impaired in the young turkey, a compensatory hypertrophy/hyperplasia of the intestine occurs, as indicated by a significant increase in length and weight of the jejunum, which at least partially overcomes the deficiency in nutrient uptake. What stimulates the jejunum to respond so quickly and what controls the increase in intestinal tissue remains unknown, but these gaps in our knowledge clearly relate to gut health and achieving the genetic potential of turkeys for growth and productivity.

(A full-length article will be submitted to a refereed journal.)

# USING FIELD EXERCISES ALONG WITH PUBLIC-PRIVATE PARTNERSHIPS TO INCREASE AWARENESS AND ACCEPTANCE OF CONTINUITY OF BUSINESS AND SECURE FOOD SUPPLY PLANS

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## ABSTRACT

In 2014, the University of Minnesota, Minnesota Board of Animal Health, and GNP Company jointly hosted a Broiler Movement Exercise which allowed Risk Analysts, USDA APHIS VS, State Veterinarians, and other animal health professionals to meet with industry veterinarians and representatives to discuss risks associated with the movement of live poultry during a Foreign Animal Disease (FAD) outbreak. The goal was to foster relationships between stakeholders, increase knowledge and understanding of live poultry movement, discuss the function of risk assessments in business continuity planning, and discuss emergency response to an FAD outbreak.

The live poultry movement activities included presentations by poultry health professionals and industry leaders, site visits for observation of normal poultry production operations, and guided participant discussions about live poultry movement in the face of a Notifiable Avian Influenza (NAI) event and the Secure Broiler Supply Plan.

The poster presentation summarizes the field exercise, exercise assessment, and lessons learned.

## BACKGROUND

Continuity of business plans being developed for the poultry industry, include the Secure Egg Supply, Secure Broiler Supply and Secure Turkey Supply Plans and serve to promote food security and animal health through continuity of business planning to support market continuity during an HPAI outbreak. These plans are centered around proactive risk assessments and make specific science- and risk-based recommendations that emergency decision makers can use to help inform their decision of whether to issue or deny permits for the managed movement of egg, broiler, and turkey industry products during an HPAI outbreak.

In order for these plans to be accurate and effective, field exercises are important both for the risk scientists take developing these plans and for animal health officials who will need to use these plans to guide animal and product movement decisions. Finally, these field exercises bring together government regulators, industry veterinarians, and scientific experts fostering understanding and cooperation, fundamental to successful navigation of a FAD outbreak event.

**Broiler field exercise purpose and design.** The Broiler Movement Exercise was a 3-day event co-planned by the University of Minnesota and the Minnesota Board of Animal Health, alongside the GNP Company. Activities consisted of presentations by poultry industry leaders, poultry health professionals, live observation of poultry operations including animal and equipment movement, and finally, guided participant discussions about potential issues surrounding live poultry movement in the event of a NAI event and the HPAI Secure Broiler Supply Plan. In addition to poultry industry leaders, risk analysts, and animal health officials, the event brought together local, national, and international influenza experts as surveillance and response activities were discussed, explored and demonstrated.

The Broiler Field Exercise was sponsored by University of Minnesota, Center for Animal Health and Food Safety (CAHFS); Minnesota Board of Animal Health (BAH); Minnesota Turkey Growers Association (MGTA); Chicken and Egg Association

of Minnesota; the GNP Company and USDA APHIS VS.

**Pre-post event survey results.** Evaluation of the Broiler Field exercise consisted of a pre- and post-event online survey. The results are presented as the overall understanding of a particular topic asked in the survey question. Twenty participants received an email request to participate in the pre-workshop survey and ten responded. In the post exercise survey, 74% of the twenty participants responded to the online survey. 100% of respondents reported that the exercise met their expectations to better understand the movement of hatching eggs, day-old chicks and broiler chickens.

Charts 1 and 2 illustrate the strengthening of participant knowledge related to product movement in the poultry industry. There was a significant increase in the number of people reporting a very good understanding of product movement in the poultry industry –increasing from only 10% pre exercise to 57% post exercise.

**Cleaning and disinfection (C&D) procedures**. Participant understanding of cleaning and disinfection procedures in a poultry operation nearly doubled during this exercise. Prior to the training, 30% of participants reported very good understanding of C&D procedures and post-exercise that number nearly doubled as 57% of respondents reported very good understanding with another 43% reporting somewhat of an understanding. Initially 10% of participants reported very little understanding, however post training, no one reported "No or very little" understanding about C&D procedures.

**Biosecurity practices in a poultry operation before the conference**. All participants in the survey had either somewhat of an understanding or very good understanding of biosecurity practices in a poultry operation. After the exercise, the number of people with a very good understanding of it increased by 17% showing increase in reported understanding in an area with an already solid knowledge base.

**Regulatory response to a HPAI outbreak.** There was little change in how people would rate their knowledge of regulatory response to a high path AI outbreak pre-exercise 90% of participants reported somewhat to very good understand of the regulatory response. Post survey that group increased to 93%.

**Poultry movement during an HPAI outbreak.** After the exercise, the percentage of participants saying they had a very good understanding of poultry movement during an HPAI outbreak almost doubled moving from 22% to 43%. The percentage saying they had little understanding decreased by 4% as well.

**Biosecurity practices during an HPAI outbreak.** Participants saying they had a very good understanding of biosecurity practices during a HPAI outbreak increased by 20% post-exercise. The percentage with somewhat of an understanding decreased by 27%, showing the exercise was effective in increasing and solidifying knowledge in this area.

## DISCUSION

This type of field exercise is valuable for poultry industry members as well as animal health and regulatory officials, both in terms of the opportunities for hands-on learning and the opportunity for in-person interaction with other animal health professionals and industry members. Broader participation from stakeholders, both in- and out-of-state, would be very beneficial. There is a need to carry out similar exercises in other regions of the country as well as for other animal agriculture commodities.

**Chart 1.**

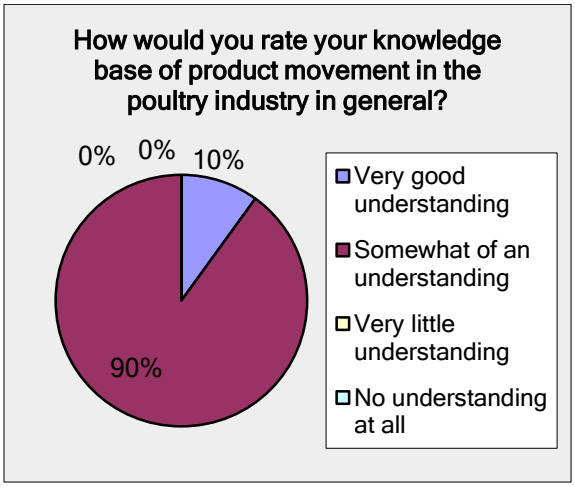
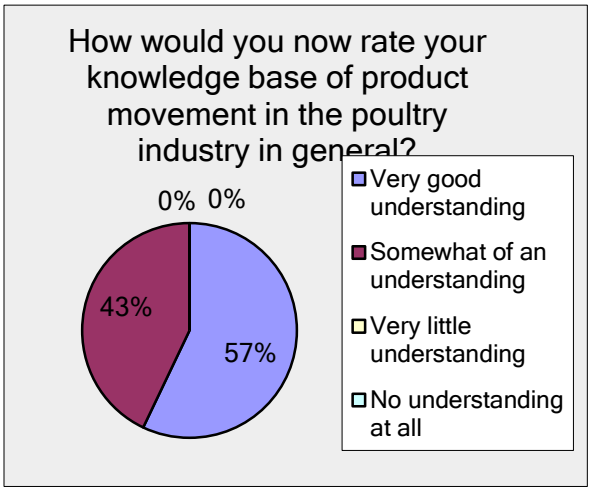



**Chart 2.**



# ISOLATION OF *ENTEROCOCCUS* AND OTHER BACTERIAL SPECIES FROM HATCH DEBRIS

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## ABSTRACT

The incidence of yolk sac infections due to *Enterococcus* infections has been increasing in recent years in the broiler chicken industry in Western Canada. Yolk sac infections, due to bacterial contamination of hatching eggs, cause major losses in the industry. Bacterial contamination of hatching eggs is likely due to hatchery and/or broiler breeder management issues, which along with yolk sac infections, may be resulting in embryonic mortality. The objective of this study was to identify infectious agent(s) associated with embryo mortality by analysis of hatch debris. Hatch debris was collected and analysed in three commercial hatcheries across Western Canada over a period of one year. A total of 3487 unhatched embryos, at varying stages of embryonic development, were examined from the hatch debris at d 21 of incubation. The majority of embryo mortality occurred during the late (34.41%) and early (19.24%) stages of incubation. Samples of yolk, for bacterial culture and identification, were also collected from randomly selected unhatched embryos at the time of the hatch debris analysis. The samples were then cultured on Columbia sheep blood agar and incubated under aerobic and anaerobic conditions at 37°C. Identification was conducted using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Bacteria isolated from these embryos were frequently *Enterococcus* spp. (~40%) and *Escherichia coli* (~30%). Of the *Enterococcus* spp. identified, more than 75% were identified as *E. faecalis*, followed by *E. faecium*. The MALDI-TOF MS identified 83.69% of bacteria to the genus level and 71.30% to the species level. From these results, MALDI-TOF MS appears to be both a rapid and reliable method of bacterial identification for both *Enterococcus* and non-*Enterococcus* spp. Bacterial species were identified in 66% of samples collected while the remaining 34% had no bacterial growth. The stages of embryonic mortality and bacterial species identified were similar in all three hatcheries studied. With the majority of bacteria isolated from unhatched embryos identified as *Enterococcus* spp., the source(s) and pathogenesis need to be investigated.

## INTRODUCTION

Enterococci are commensals of the human and animal gastrointestinal tract and opportunistic pathogens (1,2). The genus *Enterococcus* is composed of gram-positive spherical bacteria occurring singly, in pairs, or in short chains, which are nonmotile, non-spore forming, and facultative anaerobes. *Enterococcus* spp. isolated from avian species and associated with disease include *E. faecalis*, *E. faecium*, *E. durans*, *E. avium*, *E. cecorum*, and *E. hirae*. *Enterococcus faecalis* affects birds of all ages; it is a serious disease occurring in embryos and young chicks from fecal-contaminated eggs. *Enterococcus* is a normal inhabitant of the intestine of birds. Concurrent enteric infections or any condition compromising the intestinal villous epithelium allowing penetration of resident enterococci can result in septicemia, bacterial endocarditis, or both (5, 6, 7, 8, 9).

*Enterococcus cecorum* is an emerging pathogen of the broiler industry. The organism was first isolated from chicken intestinal flora in 1983 (4). *Enterococcus* spp. have been associated with brain necrosis and encephalomalacia. Egg transmission or fecal contamination of hatching eggs results in late embryo mortality and an increased number of chicks unable to penetrate through the shell at hatch. Omphalitis or enlarged yolk sacs may be seen in chicks infected at hatching. Lesions of chronic enterococcal infections include arthritis and/or tenosynovitis, spondylitis, osteomyelitis, pericarditis and perihepatitis, necrotic myocarditis, and valvular endocarditis. *E. cecorum* is a catalase-negative, alpha-hemolytic bacterium that belongs to an atypical group of enterococci that may not grow in media containing 6.5% NaCl (3). Although *E. cecorum* was previously known as a commensal in poultry, it has recently been recognized as an important pathogen of commercial broilers and broiler breeders. (12,13).

Despite the acknowledged status of the organism as a poultry pathogen, the exact mode of transmission, port of entry, and virulence mechanisms of *E. cecorum* are largely unknown. Therefore, appropriate epidemiological surveillance is a necessity for disease investigation, and has implications for preventive and control measures in an outbreak situation. Genotyping to determine the clonal diversity or to track a particular clonal type plays a pivotal role in epidemiological surveillance. It has been reported that pulsed-field gel electrophoresis provided the most reliable results with greater discriminatory power and higher reproducibility compared to the PCR-based methods for *E. cecorum* (10). Although various genomic "fingerprinting" approaches have been widely used for these purposes, one objective of this study is to analyze the genetic variability of enterococcal species and to evaluate its genetic relatedness in relation to the source of infection. Antimicrobial resistance is notorious in enterococci, and resistance genes to antimicrobial agents commonly used in chickens have been found in *E. cecorum* (11). It is possible that the use of antimicrobials may be selecting for *E. cecorum* in general or for strains which have acquired resistance determinants to frequently used antimicrobials. The objective of this study was to identify infectious agent(s) associated with embryo mortality by analysis of hatch debris.

## MATERIALS AND METHODS

Hatch debris was collected from three hatcheries from western Canada over one year period. Samples were collected over 55 breeder flocks and their age range from 27 to 60 wk. A total of 3487 unhatched embryos, at varying stages of embryonic development, were examined from the hatch debris at d 21 of incubation. Both aerobic and anaerobic conditions were employed for bacterial isolations. Bacterial species confirmation was conducted using matrix–assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technique. MALDI-TOF is widely used in routine human clinical laboratories but this technique has not been conducted for a survey like ours in the poultry industry to date.

## RESULTS AND DISCUSSION

Bacterial species were identified in 66% of samples collected while the remaining 34% had no bacterial growth. The majority of embryo mortality occurred during the late (34.41%) and early (19.24%) stages of incubation. The stages of embryonic mortality and bacterial species identified were similar in all three hatcheries studied. A total of 920 bacterial isolates were processed for MALDI-TOF MS analysis. The MALDI-TOF MS identified 83.69% of bacteria to the genus level and 71.30% to the species level. The majority of *Enterococcus* isolates identified by MALDI-TOF MS were *E. faecalis* (79.22 %) followed by *E. faecium* (11.69 %). It is evident that *Enterococcus* spp. are responsible for the majority of embryo death and further studies are needed to identify source(s) of *Enterococcus* and pathogenesis of the disease.

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# PATHOGENESIS AND CONTROL OF VIRUSES THAT ADVERSELY AFFECT AVIAN GUT HEALTH

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## ABSTRACT

Virus-induced enteric diseases are common causes of production losses in chickens and turkeys. The mechanisms by which viruses produce enteric diseases differ among the viruses that replicate in this system, with the outcome of infection determined by variables that include site of virus replication and interaction with other infectious and non-infectious factors. Regardless of the pathogenic mechanism involved, these viral diseases result in decreased efficiency of feed utilization, and this, by itself, is an important source of economic loss. Improved control of these virus-induced enteric diseases is dependent upon development of a better understanding of the pathogenesis of these infections, particularly an understanding of the interactions that occur with other infectious agents, including normal gut microflora and nutrition.

## INTRODUCTION

Several different viruses have been identified as causes of intestinal tract disease in chickens and turkeys, and several others have been associated as causes based on electron microscopic identification in tissues and/or intestinal contents of affected poultry. Virus-induced enteric infections occur in birds of all age groups but tend to predominate in young birds. Clinically, these diseases result in a broad range of outcomes ranging from inapparent, economically insignificant effects to severe and economically devastating disease.

In today's modern poultry production systems where feed represents the single largest monetary investment, these diseases invariably result in decreased efficiency of feed utilization, and this, by itself, may result in substantial economic loss. However, these virus infections often have other adverse effects on productivity, including increased mortality, decreased growth rates, decreased flock uniformity, and increased susceptibility to other infectious agents. Virus-induced damage may potentiate the pathogenesis of other infectious agents, including normal gut microflora. These infections also may result in nutritional deficiencies.

## PATHOGENESIS OF VIRUS-INDUCED ENTERITIS

Virus-induced enteric disease in chickens and turkeys may be produced by viruses that target differentiated enterocytes that cover intestinal villi (e.g. coronavirus, rotavirus) or by parvoviruses that target the crypts of Lieberkuhn. Alternatively, the avian adenovirus, hemorrhagic enteritis virus (HEV) produces enteritis by damaging cells within the lamina propria of intestinal villi.

Viruses that replicate in differentiated villous enterocytes include rotavirus, coronavirus, and astrovirus. Based on experimental studies, these viruses, by themselves, typically produce only mild disease; however, naturally-occurring enteric diseases commonly are complicated by other cofactors including other infectious agents and nutrition. Experimental challenge studies conducted in laboratory settings often fail to provide a clear understanding of the role of these viruses in naturally-occurring disease, as these studies do not duplicate field conditions (i.e. environmental, management, nutrition and microbial flora). Thus, diseases such as malabsorption syndrome, runting/stunting syndrome, poult enteritis, and poult enteritis-mortality syndrome, which likely are multifactorial in nature, generally cannot be reproduced with only viruses.

Differentiated villous enterocytes are nonproliferating cells that have both absorptive and digestive functions (3). In contrast, crypt enterocytes are undifferentiated cells that are the progenitors of villous enterocytes; these cells actively secrete fluid into the intestinal lumen. In health, the absorptive capacity of the villous enterocytes exceeds the secretory capacity of crypt enterocytes, thus net absorption occurs along the villous surface. Virus infection and consequent damage of villous enterocytes disrupt this balance by reducing the surface area of the gut mucosa (villous atrophy) and by altering the function of

individual villous enterocytes. These effects lead to a generalized reduction in digestive and absorptive capacity; functional deficits that are referred to as malabsorption and maldigestion. These functional deficits are complicated by osmotic effects in which undigested and unabsorbed feed remains in the intestinal lumen and acts to hold water. Bacterial fermentation of these unabsorbed food particles results in production of additional osmotically-active, low-molecular weight molecules that further increase the retention of water in the lumen of the intestinal tract. Unabsorbed fluids are passed down the intestinal tract to the large intestine; diarrhea results when the absorptive capacity of the large intestine is exceeded. These effects, by themselves, likely are common and economically-important sequela of intestinal infections caused by most viruses.

Virus-induced damage of villous enterocytes also may result in enteritis by potentiating the pathogenesis of other infectious agents, including normal gut microflora. While this is a likely pathogenic mechanism of virus-induced intestinal diseases of chickens and turkeys, it has received little attention. Virus-induced enterocyte damage may provide a portal of entry for other enteric pathogens such as *Escherichia coli* and *Salmonella* spp. Alternatively, virus-induced enterocyte damage may alter the intestinal luminal environment, and this may lead to perturbations of normal gut microflora.

Previous investigations have demonstrated synergistic interactions between rotavirus and enterotoxigenic *E. coli* in virus-induced enteric diseases of mammalian species (2); however, little work has been done to evaluate interactions of viruses and other infectious agents in the pathogenesis of enteritis in poultry. Turkey coronavirus has been shown to enhance intestinal colonization of enteropathogenic *E. coli* (EPEC) in young turkeys, and this combination of pathogens has been proposed as an etiological explanation for PEMS (1). It is interesting to speculate that other enteric diseases of poultry of unknown or poorly understood etiology (e. g. poult enteritis, runting/stunting syndrome of chickens) may ultimately be explained by interaction of viruses and other infectious agents.

The role of nutrition in the pathogenesis of virus-induced enteric diseases of poultry has received little attention. However, based on human and mammalian animal studies, it is likely that poor nutrition and poor feed quality contribute to severity of virus-induced enteric diseases. Studies of rotavirus infection in malnourished humans and laboratory animal models have demonstrated that malnutrition exacerbates severity and prolongs duration of clinical disease, by impairing rotavirus immune responses (4, 6). The influence of particular components of poultry diets, such as fat content, and presence of noxious compounds (e. g. rancid fat, mycotoxins) on virus enteric infections in poultry has not been examined.

The mechanism by which HEV causes intestinal disease and hemorrhage has not been conclusively determined. Unlike the enteric viruses discussed above, HEV does not replicate in villous enterocytes or crypt epithelium. Experimental studies indicate that HEV infection results in release of large quantities of proinflammatory cytokines, most importantly tumor necrosis factor, and this initiates systemic shock, leading to development of vascular lesions in the intestines (5). Such cytokine-induced vascular damage in the lamina propria leads to necrosis of villous tips and intestinal hemorrhage.

## CONTROL OF VIRUS-INDUCED GASTROINTESTINAL DISEASES

Control of viruses associated as causes of intestinal diseases of chickens and turkeys is best accomplished by maintaining premises free of these agents. Transmission of these viruses generally occurs by the fecal-oral route; flock-to-flock spread most commonly occurs mechanically via movement of people and equipment. Contaminated litter is the most likely source of infection for susceptible flocks, as well as for maintaining infection on contaminated premises. Immunization, a favored method for controlling viral infections, has been useful only for HEV infections of turkeys. Vaccination by the drinking water may be done using naturally-occurring, attenuated strains of HEV, or a closely-related virus called marble spleen disease virus.

Turkey coronavirus is successfully controlled in turkey populations based on eradication. Turkey coronavirus is readily inactivated by most common disinfectants. Successful eradication of this virus requires the identification of infected flocks. Elimination from contaminated premises then may be accomplished via depopulation followed by thorough cleaning and disinfection.

Astroviruses, reoviruses, rotaviruses, and parvoviruses are relatively resistant to inactivation and are excreted in feces in large numbers. They may survive in litter and on contaminated equipment for prolonged periods of time and this may be the primary source of infection for subsequent poultry flocks. Specific control procedures for these viruses have not been developed; control is aimed at ensuring thorough cleaning and disinfection of facilities between flocks, in order to reduce environmental contamination and degree of exposure of young poultry.

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# FULL GENOME SEQUENCES OF ORT STRAINS SHOW HIGH HOMOLOGY

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*Ornithobacterium rhinotracheale* (ORT) is a gram-negative, rod-shaped bacterium, which is frequently involved in respiratory diseases in chickens and turkeys. ORT is mostly oxidase-positive, closely related to *Riemerella anatipestifer* and more distantly related to *Pasteurella* spp. At least 18 ORT serotypes, designated A – R, have been described so far. The standard method for serotyping is the agar gel precipitation test (AGPT) using heat extracted antigens and monovalent antisera. This might have important implications for vaccination, since it is assumed, that there is no cross protection between the serotypes. Thus ORT isolates are often serotyped to make sure that the serotype of the used vaccine and of currently circulating isolates match. Serotyping might also be interesting for epidemiological studies. So far there is only limited information on differences between serotypes in regard of host preferences or virulence. However, because up to 18 different antisera must be produced in animals, which is costly and laborious, only few laboratories serotype ORT isolates. Several molecular biological methods have been tested as alternatives to serotyping, but none of these methods was able to replace serotyping until now.

In order to identify targets for serotype specific PCRs, reference strains for serotype A and I were fully sequenced and their genomes were compared to the published sequence of ORT strain DSM 15997, which belongs to serotype A and shows a cross reaction with serotype I.

The comparison showed very high homologies. Most deduced coding DNA sequences (CDS) were 100% identical; only about 65 CDS showed homologies between 80% and 95%. Additionally two small regions of 2477 bp and 1150 bp were present in reference strain A but missing in DSM 15997 and reference strain I.

In the genome of both reference strains a segment of about 50 kbp was present, which showed a homology of only between 85% and 90% between the two reference strains. Furthermore, the segment was integrated at different positions. CDS with deduced functions found on both insertions coded for three integrases, nine for proteins associated with transposons and four for proteins involved in DNA manipulation. These insertions were not present in the genome of strain DSM 15997, however there one insertion of 37.2 kbp containing 51 CDS with no homology to the insertions in the two reference strains. Several CDS coded for phage associated proteins.

The relatively high number of CDS coding for mobile genetic elements that differed between the strains suggests the possibility that mobile genetic elements might determine the serotype.

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

# RETROSPECTIVE STUDY OF HISTOMONOSIS (BLACKHEAD) IN CALIFORNIA TURKEY FLOCKS, 2000-2013

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The protozoan parasite *Histomonas meleagridis* infects turkeys as well as chickens. While mortality can be very high in turkeys, in chickens mortality usually is only slightly elevated, if at all. Two outbreaks of Histomonosis (Blackhead) in California, one affecting turkeys and the other chickens, have been described in detail (2, 9).

In the years 2000-2013, Histomonosis was diagnosed 67 times in turkey flocks in Central California by the California Animal Health and Food Safety Laboratory System. In all cases the parasite was detected by histopathology. In 61 cases Histomonosis was considered the most important diagnosis, in six cases the presence of histomonads was regarded as secondary finding.

In each year there were between one and eight cases; no clear trend was discernible. Most cases occurred in the warmer months between April and October with a clear peak in August (18 cases), which is in agreement with findings from France (1) and Germany (6). Nine cases occurred in breeder flocks, 54 cases in meat turkey flocks and 11 cases in other flocks. Diseased flocks were aged between two wk and 15 m with a median age of nine wk, about the same ages as reported before (1, 4).

Diseased turkeys had typical lesions in the ceca and livers, occasionally also in other organs. These included kidney and spleen (four cases each), bursa cloacalis and proventriculus (three cases each), as well as pancreas and crop (one case each). In turkeys as well as in chickens *H. meleagridis* causes lesions in the ceca and liver, but in both species the parasite has been detected in a variety of organs by different methods, including histopathology, PCR, immunohistochemistry and in-situ-hybridisation (2, 5, 7, 9).

Two flocks had a normal mortality, and in 23 flocks “increased mortality” was reported. In the flocks with known mortality, it ranged between 0.33% in the week before the diagnosis and more than 50% overall. Those results are more difficult to compare to European findings, since in most cases in California birds received nitarsone, which is still available as feed additive in the USA, while other drugs effective against *H. meleagridis* have been discontinued in recent years (3). So far no viable alternative to nitarsone has been identified. Alternatives to prevention using feed additives or drugs include increased biosecurity, which is difficult, since the source of infection cannot be identified in most cases, as well as possibly, in the future, vaccination (8).

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

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Shivaprasad. Systemic histomoniasis associated with high mortality and unusual lesions in the bursa of Fabricius, kidneys, and lungs in commercial turkeys. Avian Dis. 53:231–238. 2009.

# POULTRY ON-FARM NATIONAL ANTIMICROBIAL RESISTANCE MONITORING SYSTEM (NARMS): A PROGRESS REPORT

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## SUMMARY

There is not a clear understanding of the link between antimicrobial resistance in the potential food borne pathogens for humans from poultry (*Salmonella* spp. and *Campylobacter* spp.). What is even more difficult to ascertain is whether there is an effect of antimicrobial resistance of these foodborne pathogens and the level of antimicrobial resistance in the human commensal bacteria population. To date we have enrolled more than 60% of the annual U. S. broiler and turkey production. In broilers 62.5% of the bootsocks were *Salmonella* positive and 36.9% *Campylobacter* spp. positive. Turkeys were 21.8% *Salmonella* and only 1 *Campylobacter* isolate to date. Using the NARMS Sensititre panel, in broilers the greatest antimicrobial resistance in *Salmonella* spp. was to tetracyclines followed by sulfisoxazole, streptomycin and gentamicin. The broiler *Campylobacter* spp. isolates antimicrobial resistance was greatest to the tetracyclines, ciprofloxacin and macrolide, azithromycin. Antibiotic usage information was also collected from a survey completed by the production managers or veterinarians. The goal for the on farm NARMS is to not look at each year in isolation but to be able to follow trends in antimicrobial resistance and usage year after year. Results will not be written in this paper but will be presented at the meeting due to agreement with poultry company cooperators.

## INTRODUCTION

Resistance to antibiotics is considered to be one of the greatest threats to the health of both animals and humans. The Food and Drug Administration (FDA) began a program in cooperation with the U. S. Department of Agriculture (USDA) to monitor the level of resistance to many of the medically important antimicrobial agents in *Salmonella* spp. and *Campylobacter* spp. that were isolated from poultry (chickens and turkeys) in processing plants in 1996. Also during this same time the poultry industry was implementing programs to lower *Salmonella* on the final products thus lowering the number of isolates of *Salmonella* available to USDA for the NARMS program. Therefore, in 2012, FDA and USDA collaborated with The University of Georgia and The University of Minnesota to perform a pilot to environmentally sample broiler and turkey farms at processing age and culture for *Salmonella* spp. and *Campylobacter* spp. These isolates were sent to FDA for antimicrobial sensitivity testing using the Sensititre NARMS panel and serotyping of *Salmonella* and speciation of *Campylobacter*. This program was very successful and has since been expanded from the original 400 bootsocks to the current pilot projection for 1,536 samples. Also, as more and more consumer/media groups put pressure for antibiotic use information (2), the pilot was expanded to include a survey of antimicrobial growth promotion, prevention and therapeutic use on these sampled farms.

## MATERIALS AND METHODS

**Sampling approach**. The sampling plan is based on the number of slaughter plants for each broiler and turkey company, with the goal to be between 60 and 80% of the annual broiler chicken and turkey production. Farms within each complex for each enrolled company are selected at random by the production management from the farms that were within one wk of slaughter. Four bootsock samples were collected from one house on each farm with eight farms sampled at each complex (32 weekly bootsock samples). The sampling kits containing sampling instructions, sterile pre-moistened bootsocks (Solar Biologicals, Inc.) and survey were mailed to each complex. The completed antimicrobial usage survey and bootsock swabs were then overnight shipped in coolers with ice packs to either The University of Georgia or The University of Minnesota laboratory for culture. Upon arrival at the laboratory all samples had buffered peptone water (BPW) added.

***Salmonella* culture**. Following addition of BPW samples were incubated at 42°C for 24 h in Hajna tetrathionate broth (Oxoid Ltd.) and then struck onto XLT 4 agar plates (Remel Diagnostics) and incubated at 37°C for 24 h. Delayed secondary enrichment was performed for all samples negative

on the first enrichment. Isolates were serogrouped and then inoculated into DNAase/RNAase free water to have serovar determined by intergenic sequence ribotyping (ISR) as per the method of Guard et al (1).

***Campylobacter* culture**. Following the addition of BPW to the bootsocks, 1.5 mL sample was placed into 13.5 mL Bolton's broth (Oxoid Ltd.) and incubated at 42° C for 24 h then struck to Campy-Cefex agar (Remel Diagnostics) and incubated with Campy gas at 42° C for 48 h. Biochemical testing (Oxydose, indoxyl acetate and gram stain) was used to confirm identification as *Campylobacter*. Isolates were inoculated into DNAase/RNAase free water for speciation by PCR using the Rapid Finder™ *Campylobacter* Multiplex Assay beads according to manufacturer's recommendations (Life Technologies).

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# THE EFFECT OF ENVIRONMENTAL POULTRY SAMPLES ON THE PH OF TYPICAL ENRICHMENT AND PRE-ENRICHMENT MEDIA

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## INTRODUCTION

It has been known for many years that *Salmonella*, among many other organisms, is present in poultry feed and that it is a common source of contamination of poultry (2, 4). Additionally, *Salmonella* is often detected in non-host environments, such as eggshells, cloth and plastic, where it can further contaminate the environment and the animals (3, 5). There are many methods currently used to detect *Salmonella* in feed and other types of samples, the most common of which is to use an enrichment or pre-enrichment broth to ensure ideal growth conditions for the bacteria, in addition to other phenotypic and molecular methods, such as the use of XLT-4 media and PCR analysis. However, it is unknown how environmental samples contaminated with *Salmonella* might affect the growing conditions of the organism. To that same point, it is well-known that *Salmonella* is sensitive to an acidic pH, which will reduce recovery (1). Given the recent public health and veterinary concern regarding *Salmonella* in the food chain and the potential for it to be passed on to the consumer, it is critical that the methods used to isolate the organism from feed provide the most accurate and complete data possible. The objective of this study was to determine if environmental samples from a poultry house would change the pH of a pre-enrichment broth, and thus interfere with its detection.

## MATERIALS AND METHODS

**Sample procurement and processing**. Samples were recovered from a commercial broiler hatchery and farms associated with the University of Georgia in Athens, GA at two independent time points. Samples included turkey litter, broiler litter, boot covers that had been used in turkey and broiler pens, fluff, and eggshells. To process the samples, litter and egg shells were run through a splitter twice before being finely ground in a blender. Fluff was run through the splitter twice prior to processing. Boot covers were cut into small pieces and mixed together before sampling. All samples were weighed into 10g samples.

**Media analysis**. The types of media analyzed were as follows: M-9, Lactose broth (LB), Rappaport-Vassiliadis broth (RV), universal pre-enrichment broth (UPB), tetrathionate broth (TTH), and buffered peptone water (BPW). 90 mL of each media were dispensed into sample bottles, the initial pH was taken and recorded, and the weighed samples were subsequently added to each type of media. The pH of each media at time 0, immediately after the samples were added, was also taken and recorded. Samples were incubated at 37ºC for one h (at 42ºC for RV and TTH) and the pH was then taken. The samples were then replaced in their respective incubators. The pH was recorded at 18, 24 and 48 h from time 0.

## RESULTS AND DISCUSSION

The initial pH of all tested media (with the exception of RV), prior to sample addition, was an average of 6.9. The initial pH of the RV was 5.1. By media, the greatest pH changes were in LB and TTH, with an average pH drop of 2.4 and 1.7, respectively. The least amount of pH change was observed in RV and UPB, with average drops of 0.2 and 0.1, respectively. The most ideal media for environmental samples was BPW, as the pH was consistently between 7.2 and 6.5. The pH within sample type varied by media, and no clear correlation to pH level and environmental sample type was established in this study.

The results from this study reveal how the buffering capacity of different media can vary, and how this could influence *Salmonella* detection, as acidified environments may reduce the numbers of the organism detected. Based on these results, the best type of pre-enrichment media is BPW for environmental poultry samples, due to the lack of change in pH over time and the neutrality of the media after 48 h of incubation. Maintaining a strong

buffering effect in the media, as seen with BPW, can prevent significant pH changes and help reduce *Salmonella* detection loss.

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# POSTMORTEM FINDINGS IN LAYING HENS HOUSED IN NON-CAGE SYSTEMS IN CALIFORNIA AND IOWA: PRESENCE AND SEVERITY OF LESIONS AND THEIR RELATIONSHIP TO STAGE OF LAY

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## SUMMARY

The egg industry has started moving away from conventional cages towards alternative housing systems for laying hens, including non-cage housing systems (i.e. barns, aviaries). Although concerns about hen welfare drive these changes, the frequency of adverse health conditions in non-cage hens in the US is unknown. This study aimed to evaluate the health of non-cage laying hens in two of the largest egg producing states, California and Iowa, by conducting postmortem examinations of mortalities to assess lesions and their severity during different stages of the laying cycle.

Laying hens (n = 308) from organic and non-organic non-cage farms were examined postmortem during early (16 to 34 wk of age), mid (45 to 55 wk of age), and late (66 to 80 wk of age) lay. Twenty-five lesions, selected for known association with hen welfare, were investigated and scored by frequency and severity. Lesion frequency was then compared statistically by stage of lay.

Vent cannibalism, keel bone deformation, and/or abnormal beak length were observed in 49.3%, 48.7%, and 40.2% of hens, respectively. Beak length and enteric disease lesions, when identified, were more often severe. Vent cannibalism and footpad dermatitis were seen most frequently during mid lay, while septicemia was seen most frequently in early lay in Iowa and late lay in California.

Although genetics, farm management practices, and environmental factors could have affected these results, the information can be used to better understand hen health in non-cage housing systems and to identify potential interventions to reduce hen welfare problems.

# RESEARCH UPDATE ON OUTBREAKS OF H5N8 AND H5N2 HIGHLY PATHOGENIC AVIAN INFLUENZA IN THE U.S.

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**SUMMARY**

In December 2014, following the outbreak of H5N2 highly pathogenic avian influenza (HPAI) in British Columbia, Canada, near-simultaneous isolations of HPAI virus (HPAIV) were recovered from the northern pintail (NOPT) duck and captive gyrfalcons in Washington State. Two different HPAIV subtypes were identified in these birds, H5N2 in NOPT and H5N8 in gyrfalcon. In January 2015, additional HPAIV isolations were reported in California, Idaho, Oregon, Nevada, Utah and Washington. These H5 isolations were confirmed in wild birds, backyard poultry and a commercial turkey operation. During this time period an additional HPAIV subtype, H5N1, was also reported in a green winged teal in Washington State.

Sequence analysis of these HPAI viruses has confirmed that all are related to a H5N8 HPAIV isolated in South Korea in January 2014. During the original outbreak in South Korea, more than 29 farms were confirmed positive which affected chickens, ducks and geese. These H5N8 HPAI viruses evolved from the Asian H5N1 HPAI-lineage that was first recovered in Guangdong, China, in 1996. These H5N8 viruses belong to Asian-H5 clade 2.3.4.4 and contained only Eurasian gene segments. Subsequently in November 2014, the H5N8 viruses were detected in commercial turkeys, chickens and ducks in Germany, Netherlands and United Kingdom, respectively. The detection of H5N8 in Western Europe implicates migratory wild birds as likely source of virus spread out of Asia.

In North America, the H5N8 HPAI viruses have apparently also spread quickly along migratory waterfowl pathways including the Pacific flyway. However, the viruses have apparently also reassorted in wild birds with low pathogenic avian influenza viruses to generate these new H5Nx (H5N2, H5N1) HPAIV isolates. Further investigation and characterization of these HPAI viruses is ongoing.

# ANTIMICROBIAL RESISTANCE OF *CLOSTRIDIUM PERFRINGENS* ISOLATES OBTAINED FROM COMMERCIAL ONTARIO BROILER CHICKEN FLOCKS

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## ABSTRACT

*Clostridium perfringens* is responsible for necrotic enteritis, an economically significant disease that occurs in broiler chickens. Although the exact role of *netB* in disease development is not known, this gene is considered an important virulent factor in disease pathogenesis. Our objectives were to determine the proportion of resistant *C. perfringens* isolates obtained from a representative sample of Ontario broiler flocks for 11 antimicrobials of importance to veterinary medicine, and to determine the association between antimicrobial resistance and presence of the presumably virulent *netB*.

Five pooled samples of cecal swabs from 15 birds/flock from 231 randomly selected flocks were anaerobically cultured using standard techniques for *C. perfringen*s. Polymerase chain reaction (PCR) was used to test isolates for genes encoding various toxins (α, β, ε, ι, enterotoxin, and *beta2*), and real-time PCR was used to test isolates for *netB*. Minimum inhibitory concentrations were determined using the microbroth dilution method. STATA was used to create unconditional mixed logistic regression models with flock as a random effect to identify associations between antimicrobial resistance and presence of *netB.*

*Clostridium perfringens* was isolated from 181 of 231 flocks (78.4%; 95% CI: 73.0 to 83.7%). All isolates were type A, except for one type E. *NetB* was identified in 71 of 231 flocks (30.7%; 95% CI: 24.7 to 36.7%) and in 169 of 629 *C. perfringens*-positive isolates (26.9%; 95% CI: 23.4 to 30.3%). Isolates were resistant to oxytetracycline (64.5%; 95% Confidence Interval (CI): 61.7 to 0.68.2%), bacitracin (64.2%; 95% CI: 60.4 to 67.9%), erythromycin (62.3%; 95% CI: 15.7 to 21.8%), tetracycline (62.2%; 95% CI: 15.6 to 21.7%), ceftiofur (49.4%; 95% CI: 45.5 to 53.3%), clindamycin (21.1%; 95% CI: 0.18 to 0.25%), tylosin tartrate (18.4%; 95% CI: 15.6 to 21.7%), and penicillin (2.0%; 95% CI: 0.00 to 0.01%). *NetB* was positively associated with resistance to oxytetracycline (OR = 13.9, 95% CI: 5.1 - 37. 6, p = 0.001), and tetracycline (OR = 21.1; 95% CI: 7.7 – 58.2, p = 0.001), and negatively associated with resistance to clindamycin (OR = 0.3; 95% CI: 0.1 – 0.9, p = 0.027), and bacitracin (OR = 0.31; 95% CI: 0.1 – 0.6, p = 0.002). A high proportion of isolates were resistant to oxytetracycline, bacitracin, erythromycin, or tetracyclines, a moderate proportion of isolates were resistant to ceftiofur, and a low proportion of isolates were resistant to clindamycin, tylosin tartrate, or penicillin. Although associations were found between antimicrobial resistant isolates and *netB*, the clinical significance of this finding is not yet known.

# COCCIDIOSIS PREVENTION AND CONTROL IN "SPECIALTY BROILERS"

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## SUMMARY

This case report summarizes on-farm findings and interventions that were employed through Platinum Brooding® to improve flock performance and reduce disease challenges.

The importance of optimum management cannot be overemphasized in terms of overall health and ability for a bird to reach its genetic potential. Deficiencies in management can result in poor performance and increased severity of infectious disease. In this case, a farm that grows specialty broilers (Taiwanese chickens) has a history of re-occurring coccidiosis and poor performance parameters (small, poor-doing, lame birds, with high mortality) over the past three cycles. This case study emphasizes the importance of measuring on-farm parameters to assess management deficiencies and prevent disease.

Platinum Brooding is a program that has been developed to provide a comprehensive review of critical brooding and management factors. The Platinum Brooding checklist is used as a guideline and provides the information necessary to identify and correct deficiencies in management. The checklist includes measurements pertaining to chicks, litter, feed, space, air, water, feed, light and sanitation. In particular, the checklist emphasizes the importance of crop-fill. It has been widely accepted that crop-fill at 24 h post-placement should be greater than 95% to consistently reach body weight, uniformity and performance targets. This case demonstrates how applying the Platinum Brooding Checklist and monitoring these key management parameters can solve recurrent disease and performance issues.

## ACKNOWLEDGEMENTS

The author would like to acknowledge the help of Stewart Ritchie and Mike McIlwee of Canadian Poultry Consultants Ltd., Abbotsford, British Columbia

# MAPPING OF THE SPATIOTEMPORAL INTERSECTION OF AVIAN INFLUENZA RESERVOIRS IN CALIFORNIA AS A MECHANISM FOR GUIDING SURVEILLANCE

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## SUMMARY

Past surveillance efforts for avian influenza viruses (AIv) in commercial poultry have typically neglected spatiotemporal correlation with known reservoirs for the virus, primarily wild waterfowl. In addition, association of AIv in less common reservoirs that could potentially serve as mixing vessels for the virus, such as feral swine is absent from the literature. An additional component to AIv disease dynamics not typically addressed in previous research is the spatiotemporal variability of both natural and man-made wetlands such as rice fields. A comprehensive spatiotemporal cross-species AIv surveillance effort may elicit new insight regarding AIv transmission and facilitate more targeted surveillance efforts in commercial poultry and waterfowl. In the first step of this project, we performed a mapping study to evaluate the spatiotemporal intersection of commercial and backyard poultry operations with migratory wild waterfowl flyways, feral swine, natural wetlands, and rice fields in California. A polygon-based proximity analysis was implemented to identify spatial association.

## INTRODUCTION

Recent detection of H5N8 and H5N2 strains of Highly Pathogenic Avian Influenza (HPAI) in British Columbia, Washington, Oregon and California in December 2014 underscores the importance of active AIv surveillance to protect commercial and backyard poultry in California. The primary reservoir for AIv – migratory waterfowl – is thought to introduce the virus during overwintering periods to naïve populations that exist near roosting and feeding sites (1, 3, 4, 5). The locations of these sites are highly dependent on the presence of wetlands and flooded cropland, and thus represent identifiable and deterministic regions of high risk for AIv transmission. Proximity of these sites to commercial and backyard poultry represent the highest risk for transmission to domestic species, and thus the highest risk for large-scale AIv epidemics. Additionally, proximity to feral and captive swine represent high risk for harboring and reassortment of influenza viruses (6, 7). Traditional surveillance for AIv in high risk wildlife and backyard poultry is primarily passive and hence relies on submissions from the public. In contrast, the commercial poultry industry has a robust system of surveillance codified by the NPIP. However, these approaches do not take into account factors including seasonality and geography. Identification of the spatiotemporal patterns of migratory waterfowl and their association with known susceptible species facilitates a more directed, active approach to AIv surveillance.

## MATERIALS AND METHODS

Location of wetlands, crops, poultry and swine within California were obtained from various online governmental and non-governmental sources as shapefiles. All were imported into ArcGIS 10.2 for processing. A variably-sized buffer was placed around susceptible species (poultry, swine) and overlap with wetlands and flooded crops was quantified and ranked. Various proximity and hotspot analyses were performed.

## RESULTS AND DISCUSSION

In order to develop a comprehensive understanding of water fowl habitat relative to domestic poultry, mapping of commercial farms, backyard poultry, feral swine and waterfowl habitat was integrated with literature reviews to understand the behavior, distribution and range of movement. Results show highly variable space-time clustering, potentially associated with risk of AIv transmission. Targeting of these regions for surveillance efforts may lead to more efficient, earlier detection of AI viruses in California.

## CONCLUSION

Spatial and temporal mapping of geographical areas where waterfowl interface with domestic poultry was performed in order to better target AI surveillance efforts in California. The first step in understanding the risk of AI to domestic poultry is to understand the relationship between the agent, host, and the environment. An understanding of this classic epidemiologic triad is essential toward breaking the transmission cycle of any infectious disease. Consequently, epidemiological tools including GIS and spatial statistics are essential toward understanding risk. In this project, the spatiotemporal intersection of commercial and backyard poultry operations with migratory wild waterfowl flyways, feral swine, natural wetlands, and rice fields in California was identified to assess risk for AIv transmission. Future directions include use of weather radar to precisely identify waterfowl roosting sites (2) and real-time processing of satellite imagery to map temporal changes in wetlands and flooded croplands.

## ACKNOWLEDGEMENTS

Central Valley Joint Venture and Ducks Unlimited for providing shapefiles of wetlands and crop locations within California.

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# APPLICATIONS OF NEXT-GENERATION SEQUENCING IN VIRAL AND BACTERIAL GENOME SEQUENCING

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**SUMMARY**

The advent of Next Generation Sequencing (NGS) technologies offers unprecedented opportunities for the advancement of genomic research specifically enabling scientists to do high volume sequencing. With NGS full genome sequencing (no gaps) of viruses and bacteria is no longer a formidable task and such projects can be done not in years but rather in months, weeks and in some cases in days.

This platform of technology has revolutionized the landscape of microbiology and infectious disease. I will discuss the different platforms of Next Generation DNA Sequencing and reveal our development of doing a single full viral and bacterial genome sequencing (no gaps) bringing the completion time from months to weeks and in some cases from weeks to days. I will also discuss how we develop a protocol to finish 50 bacterial genomes in 6 months and are heading to finish 100 bacterial genomes in less than 8 months of time. NSG has already started moving into human clinical laboratory in recent years and soon be applied in veterinary clinical setting as well.

(The full length manuscript will be submitted for publication.)

# CHARACTERIZATION OF NOVEL NANOPARTICLE BASED VACCINE FOR AVIAN INFLUENZA

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## SUMMARY

Influenza (bird flu), is a devastating poultry disease causing seriously economic lose in poultry industry as well as posing public health concerns (1). Vaccination is the most effective strategy to combat avian influenza (AI). Vaccine designing, however, is challenging due to the highly mutable genome of influenza virus (3). Peptide nanoparticle technology has been demonstrated as a powerful platform for the delivery of epitopes to elicit strong immune response, providing promise in the development of universal influenza vaccines (2). Particularly, in this study, four self-assembled nanoparticle peptides (SAPNs) were designed to organize AI M2e peptide as a tetramer in addition to include conserved helics A, C and the cleavage peptide of hemagglutinin as well as chicken specific T cell epitope (HTL), respectively in each construct. Structural organizations of the epitopes on the SAPNs are to best display them in their native conformation as in the virus to induce immune response. Groups of specific pathogen free (SPF) chickens were immunized intramuscularly with these four constructs for three times with two wk apart between each vaccination. Specific antibody responses to each of the vaccine constructs were tested by ELISA. Vaccinated chickens exhibited increased Ig Y responses for each of the constructs as compared to a non-vaccinated group. Plaque reduction assay was performed to test neutralizing antibody.

ELISA results suggesting that tetra-M2e-HTL and tetra-M2e-Helices A construct induce strong antibody titer in serum of vaccinated chicken comparing to control group. Plaque reduction assay revealed that Helix C construct induced neutralizing antibody blocked H5N2 infection on MDCK cells in vitro.

In the future, T cell mediated immunity induced by these construct will be tested by lymphocyte proliferation and flow cytometry. Construct inducing best immune response will be selected to further modification to improve immunogenicity. Finally, they will be applied to immunize chickens in challenging assay to test its promising application in poultry industry.

The results suggest that the SAPNs show promise as a potential platform for a development of a vaccine against AI. Further studies are in progress. High path challenge studies will be carried out in near future after selection of the nanoparticles construct with higher immunogenicity and neutralizing antibody responses.

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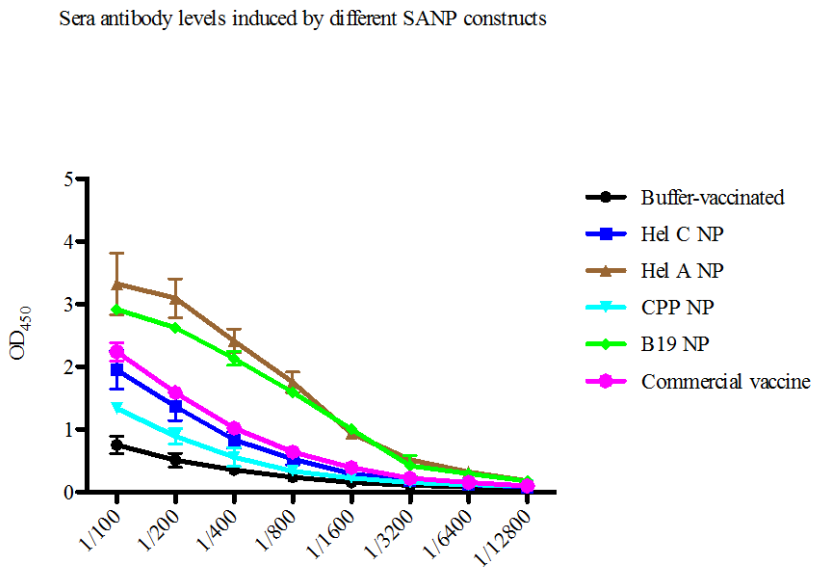


Figure 1. Serum antibody titer at 2wks post 2nd boost vaccination by ELISA test

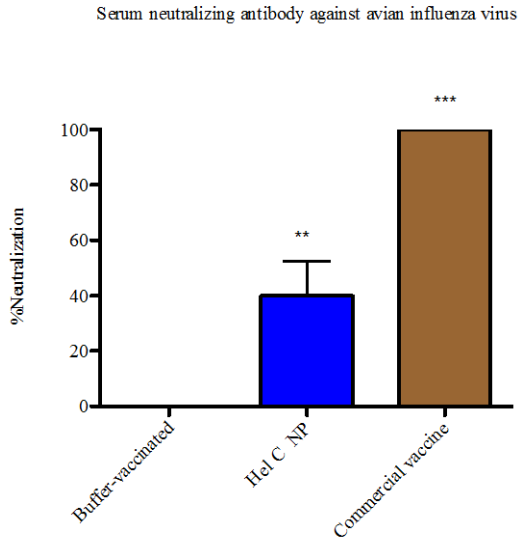


Figure 2. Reduction of infection of H5N2 virus in MDCK cells by sera from Hel C NP-vaccinated birds

# *ESCHERICHIA COLI* IN TURKEYS: A LONGITUDINAL STUDY OF *ESCHERICHIA COLI* VIRULENCE FACTORS IN TURKEYS

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## ABSTRACT

Virulence factors of *Escherichia coli* obtained from samples of two genetic lines of two commercial light hen turkey flocks were compared. Flock A was raised for teaching purposes at the North Carolina State University College of Veterinary Medicine, while flock B was raised on a North Carolina commercial farm. Each flock was comprised 50% of hybrid turkeys and 50% of Aviagen-Nicholas turkeys. For *E. coli* culture, culturette swabs ($n$ = 345) were used to sample healthy birds, sick birds, and the environment of each flock from placement through processing. Fecal samples ($n$ = 50, all positive for *E. coli*) and environmental swabs ($n$ = 71, 28 positive) were cultured weekly, while tissues and organs were cultured as needed at necropsy of dead and cull birds, and at processing ($n$ = 224, 178 positive). Ten cloacal swabs—five from each breed—were taken from each flock at processing. A total of 256 samples (74.2%) were positive for *E. coli* based on culture and biochemical tests. Positive cultures were characterized by molecular methods to determine the presence of virulence genes *cvaC, iroN, ompTp, hlyF, etsB, iss, aerJ, ireA*, and *papC*. With the exception of *ireA* and *papC*, all of these genes are linked to the large virulence plasmids that characterize avian pathogenic *E. coli* (APEC).

Virulence genes occurred frequently among the *E. coli* isolates. In flock A, 126 of 170 isolates (74.1%) possessed at least one virulence gene, and 105 (61.8%) possessed four or more virulence genes. In flock B, 61 of 86 isolates (70.9%) possessed at least one virulence gene and 50 (58.1%) possessed four or more virulence genes. *E. coli* isolates from flocks A and B averaged 4.8 and 4.1 virulence genes, respectively.

The prevalence of each virulence gene varied slightly between flocks. Gene *iroN* dominated in both breeds of flock A at 14.3% of the 811 genes identified in the 170 flock A cultures. In flock B, virulence gene *ompTp* and toxin gene *hlyF* tied for overall highest prevalence (each 13.9% of the 353 virulence genes detected in the 86 flock B cultures). Chromosomal adhesin gene *papC* was the least prevalent virulence gene in both flocks (1.8% flock A, 4.2% flock B) and in both breeds (0.4% of hybrid flock A genes, 4.5% of Nicholas flock A genes; 3.5% of hybrid flock B genes, 4.8% of Nicholas flock B genes).

(Full manuscript will be submitted for publication in a peer-reviewed journal.)

# THE SECURE EGG SUPPLY PLAN: BUSINESS CONTINUITY PLANNING FOR A HIGHLY PATHOGENIC AVIAN INFLUENZA OUTBREAK

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## ABSTRACT

The Secure Egg Supply (SES) Plan was developed through a collaborative effort between government, industry, and academic partners in order to facilitate continued movement of uninfected egg products in the event of a highly pathogenic avian influenza (HPAI) outbreak in the United States. Proactive risk assessments performed by analysts at United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), Center for Epidemiology and Animal Health (CEAH) serve as the bases for determination of permit requirements for movement of eggs and egg industry products from infected but undetected flocks. Entry and exposure assessments were performed for the following commodities: pasteurized and non-pasteurized liquid eggs, washed and sanitized shell eggs, nest run shell eggs, shells and inedible eggs, hatching eggs, day-old chicks and manure. To move eggs, producers must then complete a series of steps to be issued a permit by the Incident Commander's designee. Producers are encouraged fulfill preparatory steps prior to an outbreak to expedite permit receipt when needed. First, traceability information (premises ID, Global Positioning System (GPS) coordinates, etc.) must be made available. Next flock production parameters must be within normal limits. Product-specific biosecurity measures must be in place for premises, people, flock, pest control, equipment and egg-handling materials, feed and water, and manure removal. An epidemiological assessment must be found to be acceptable by the issuing official. Prior to movement and depending on product negative real-time reverse transcriptase polymerase chain reaction (RRT-PCR) may be required on the one to two days preceding product transport. The SES Plan is currently in the implementation stage; partners in academia, industry and government are performing outreach activities to encourage adoption and modification of the plan by individual states and regions to best suit their industries' needs.

## INTRODUCTION

In the event of an HPAI outbreak in the US poultry industry, regulatory measures will be taken to contain and eradicate the disease expediently. Limitations on movement of eggs and egg industry products into, out of, and within an HPAI Control Area will be applied. However, these restrictions directly oppose the egg industry's need to continue to move uninfected product to commerce to avoid storage/disposal and economic crises, and to supply eggs to consumers. With the ever-present risk of an HPAI outbreak in the US, whether introduced through trade or human travel, wild bird migration, or re-assortment of previously circulating influenza viruses leading to emergence of a highly pathogenic strain, business continuity planning is of great importance to protect farmer livelihoods and animal welfare, and to avoid disruption of the food supply.

## MATERIALS AND METHODS

The SES Plan was developed by a collaborative public-private-academic team (Egg Sector Working Group) which consists of members from the University of Minnesota Center for Animal Health and Food Safety (CAHFS), Iowa State University Center for Food Security and Public Health (CFSPH), United Egg Producers (UEP), egg sector veterinarians and officials, state officials, and risk analysts from the USDA, APHIS, VS, and CEAH. The initial step in SES Plan development was a literature review of studies on HPAI transmission dynamics, product-specific risk assessments (RAs), and management of non-infected premises. Examination of previous research findings served as a basis for proactive RAs on the survivability and transmissibility of avian influenza in eggs and egg industry products. Entry and exposure assessments

were performed for the following commodities: pasteurized and non-pasteurized liquid eggs, washed and sanitized shell eggs, nest run shell eggs, shells and inedible eggs, hatching eggs, day-old chicks and manure (completion of the latter is currently in progress). Risk analysts combined data obtained in the literature review with the following considerations: characteristics of HPAI in infected hens and HPAI spread within an infected table-egg layer flock; likelihood of eggs laid by HPAI-infected chickens being contaminated with virus; variability in detecting HPAI infection with various active surveillance protocols given the prevalence of HPAI in pools of daily mortality; and the frequency of product movement. After taking into consideration product-specific and general biosecurity, specified product movements were assigned risk designations. Additionally, normal production and mortality parameters were established through expert opinion and published research to develop surveillance guidelines and testing for HPAI via RRT-PCR. RAs and mortality/production parameters serve as the bases for development of voluntary preparedness components and permit requirements for movement of eggs and egg industry products from infected but undetected flocks into, out of, and within an HPAI Control Area.

## RESULTS

In the event of an HPAI outbreak, producers who wish to obtain a permit must fulfill a series of steps. Premises traceability information must be provided to the State coordinator. Next, flock production parameters must be normal. Daily flock observation for abnormal clinical signs, egg production rate, and mortality rate must be performed by the producer. The normal production parameters are defined as daily mortality of less than three times the past seven d average or less than 0.03 percent. Daily surveillance consists of one RRT-PCR test for each pooled sample of five dead or euthanized sick chickens or 11 dead or euthanized sick chickens per 50 dead chickens from each house on the premises. A minimum of five dead chickens or 11 dead chickens from daily mortality or from euthanized sick birds from each flock must be tested each day. Biosecurity and cleaning and disinfection steps must be in place for the transporting truck and driver; product-specific steps have also been mandated for premises, people, flock, pest control, equipment and egg-handling materials, feed and water, and manure removal. The aforementioned epidemiological assessment must be completed for the farm of origin, with no findings indicating dangerous contacts with Infected Premises. Finally, a product-specific one- to two-day hold on movement must be respected, pending the analogous receipt of one or two negative daily RRT-PCR tests from the flock of origin.

The Voluntary Preparedness Requirements were developed to allow for expedited receipt of a movement permit in the event of an HPAI outbreak. Essentially, producers complete portions of permit requirements proactively and egg farms preapproved by the State Animal Health Official (SAHO) or the USDA Assistant District Director will be subject to audited minimum biosecurity standards. Nonparticipatory producers will face additional delays in moving their products while animal health regulatory officials designated by the Incident Commander ascertain premises' biosecurity practices, determine exposure to dangerous contacts with Infected Premises, and conduct daily surveillance of flocks in the Control Area. Voluntary pre-enrollment consists of the following:

- Compliance with an audited 45-measure biosecurity checklist consisting of implementations that would reduce the risk of introducing HPAI virus onto the farm,
- Provision of location/traceability verification - premises ID, GPS coordinates,
- Training on completion of the epidemiological questionnaire and entry of flock data into the secure SES data portal, and
- Training on procedures to collect and submit samples for the active surveillance program using RRT-PCR.

## DISCUSSION

The SES Plan is currently in the outreach and implementation phase of its development. Some States have already developed and adopted memoranda of understanding or other mechanisms to implement the SES Plan during an outbreak. During the 2015 calendar year, regional meetings are planned to bring together animal health officials, academic and industry representatives from the Pacific, Southeast, and Northeast States and Minnesota. Key stakeholders in States of interest were identified through SES team members' contacts and referrals provided by these initial contacts. Meeting activities include presentations by HPAI subject matter experts, table-top exercises, and movement exercises at farm and processing facilities. The SES team is also working to develop website material streamlined for the producer community and branding the SES name with the assistance of graphic design personnel.

Further information and materials can be found at http://secureeggsupply.com. Funding for

development of the SES Plan was provided by USDA APHIS and the American Egg Board. Further funding for outreach and implementation of the plan has been provided by USDA APHIS.

# ATYPICAL GROSS LESIONS ASSOCIATED WITH *ASPERGILLUS FUMIGATUS* IN ORGANIC, CAGE-FREE COMMERCIAL LAYERS

G.A. Lossie, P. Wakenell, and Y. Sato

## INTRODUCTION

Aspergillosis is caused by *Aspergillus fumigatus*, however the following fungi can play a role in the disease process: *Aspergillus niger*, *Aspergillus glaucus*, *Aspergillus nidulans*, and other *Aspergillus* spp. (1). Aspergillosis is usually confined to the pulmonary system and is known as brooder pneumonia in young poultry (2). Less common sites of infection include the eye, brain, skin, joints, and viscera (2). Clinical signs depend on the infective dose as well as spore distribution, pre-existing conditions, and the immune status of the host (1). This case report demonstrates an interesting case of chronic aspergillosis in sexually mature adult birds.

## CASE HISTORY

Two groups of live and dead, cage free, organic, Hyline Browns were presented to Purdue ADDL for necropsy. Birds were 25 wk in age from a flock of approximately 20,000 birds. The flock had a history of increased mortality over a two wk period with birds initially exhibiting varying degrees of torticollis. Birds went on to exhibit respiratory signs.

At gross necropsy, all three live birds exhibited moderate to severe torticollis. One of the dead on arrival birds had a 3cm x 1.5cm x 1cm focal nodule filled with caseous material present within the interclavicular air sac. Two out of four dead birds had caseous plugs located at the tracheal bifurcation. One bird had an off white to yellow 2-3 mm diameter circular nodule within the lung.

Histologic examination of the above noted specimens revealed the following: mild subacute lymphocytic tracheitis, heterophilic laryngitis, and necrotizing airsacculitis. Microscopic lesions were nonspecific and did not suggest a fungal etiology.

Virus isolation was performed on the brain, lung, and trachea. Results of virus isolation were negative. Avian influenza antigen capture tests were performed on the three live birds and yielded negative results. Fresh air sac and lung were submitted to bacteriology for culture. The air sac was positive for *Aspergillus fumigatus* and the lung was positive for *E. coli*. (Note: Fungal culture results were not obtained until after the field visit.)

## FOLLOW UP

A field visit to the location where the birds originated was conducted as elevated flock mortality continued. Field necropsy of a total of 39 dead and culled birds revealed that 9 of the 39 birds had large fibrous granulomas present in the left and right caudal abdomen. Caudal abdominal granulomas measured approximately 3cm wide, 3cm deep, and 5cm tall. Granulomas on cut section were hollow containing black to green fungal plaques. One bird exhibited flat fungal plaques at the tracheal bifurcation which measured 2 cm x 1.5cm on the left and 1.5cm x 0.7 cm on the right. Samples were collected for histological examination. Histology revealed the presence of numerous, septate, dichotomously branching fungal hyphae with parallel sides within and along the periphery of chronic granulomas. Hyphae measured approximately 2.5 µm in width and ranged from 5.7µm to 50µm in length. Colorless to pale green conidiophores measuring 25µm to 30µm in diameter were present adjacent to areas of active inflammation. Conidiophore terminal vesicles were tipped in upward facing phialides that were parallel to the axis of the conidiophore.

Histopathologic diagnosis of the second set of samples was obtained at the same time as the bacteriology culture noted above. A subsequent visit to the farm revealed that mortality was decreased but remained elevated, with fewer birds demonstrating clearly visible fungal granulomas at necropsy. Multiple birds had air sac abscesses filled with off white to yellow caseous aggregates. Further history from the flock manager revealed that the pullet barn that these birds were housed in had developed a large leak in the roof with approximately half of the barn having damp and wet litter.

## DISCUSSION

This case is unique in that the initial sign observed was torticollis, without respiratory involvement. The marked size and caudal distribution of fungal granulomas contributes to the uncommon nature of this case.

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# MANAGING COCCIDIOSIS IN BIRDS RAISED WITHOUT ANTIBIOTICS

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## INTRODUCTION

For poultry producers to make a profit they must have healthy, uniform sized birds, make sure there are no real or assumed human health concerns that are associated with limiting antibiotic resistance, all while formulating diets on a least cost basis. Traditionally, antibiotics such as Ionophores (anticoccidial), bacitracin methylene disalicylate, tylosin phosphate and virginiamycin maintain healthy birds resulting in growth promotion and thus increased profit. Even though Ionophorus antibiotics are not used in human medicine they are also grouped with all antibiotics. The ban of feeding antibiotics to broilers in EU, and the general increased awareness of consumers has increased the demand for "antibiotic free" poultry products. Thus, there is a need for optional products to antibiotics both in coccidiosis control and bacterial related intestinal health. Some of these products are direct fed microbials, prebiotics (MOS), essential oils, saponin or sapogenines (yucca and/or quillaja), acidifers, and physically activated vitamin C combined with organic acids. Principally, the essential oils and saponins have demonstrated the most significant anticoccidial activity. As with the antibiotics, these products must work in today's poultry production environment where feed prices are often high, alternative feed ingredients are routine, marginal coccidiosis control is common (drug resistance and or vaccination issues), heavily built-up litter is used, and the house environment highly variable. All of the afore-mentioned can lead to intestinal health issues which must be managed by these products.

## DISCUSSION

The goal of any anticoccidial program is to control severity of coccidiosis and also attempt to regulate when the primary damage will occur. The amount of damage is related to the species, amount, frequency and timing of exposure. Coccidiosis, even mild cases, has a negative impact on production with losses in feed conversion, weight gain, uniformity, pigmentation, and increased mortality. Coccidiosis continues to be the most frequently diagnosed disease in poultry. Almost all commercial poultry will become infected with coccidian; that's some ~9 billon coccidia infected chickens each year in the USA. Coccidia are protozoan (*Eimeria* spp.) parasites that infect the intestine or ceca of poultry. The level and species of coccidia will vary with anticoccidial programs, management, and bird age

Poultry coccidiosis is controlled by the use of prophylactic feeding of anticoccidial drugs (no ionophorous antibiotics in antibiotic free production) or vaccinating with live coccidial vaccines. Drugs work directly on the coccidia. Degree of anticoccidial activity depends on efficacy and sensitivity, which vary greatly. Anticoccidials are broadly divided into synthetic (chemical) and ionophores. In the US, currently the only approved non antibiotic anticoccidial drugs are: diclazuril, clopidol, amprolium, robenidine, zoalene, decoquinate, and nicarbazin. Both drug and vaccination programs also rely on immunity development. In order to predict coccidiosis control and immunity development, performance and related oocysts litter/fecal numbers are valuable tools. *Eimeria* are very immunogenic. With each cycle of coccidia in the host, immunological protection increases. The development of self-limiting immunity, which eventually protects a flock, is a very critical objective for a coccidiosis control program, whether vaccination or an anticoccidial drug program. Fully sensitive chemical drugs limit oocysts shedding and related immunity development. The lack of full immunity once the chemical is removed influences subsequent degree and timing of coccidia development. Ionophores and partially resistant chemicals work similarly with partial direct control and regulated immunity development. Both of these traits allow some oocysts to be shed over the course of the growout with accompanying immunity development. Generally, oocysts shedding with this type program increases with a peak approximately d 28-35. However Chapman (1) demonstrated that full immunity to most anticoccidial drugs take at least 6 to 7 wk.

The major concern with anticoccidial drugs is development of resistance. Resistance to some degree has developed to all drugs (2).Rotation and resting (not using for extended periods of time) slows resistance development. However with many of these

drugs once resistance has developed it is very persistent / stable and years of non-use are needed to see a significant change. Anticoccidial sensitivity of coccidia isolated from poultry houses can be determined by anticoccidial sensitivity tests. ASTs are very useful in attempting to predict the control program that will have the most useful/ sensitive drugs.

Due to the increasing demand for antibiotic free birds and concerns of resistance issues with anticoccidial drugs, the use of coccidiosis vaccination has grown tremendously in the last few years. The only method to produce a truly drug-free bird is through the use of coccidia vaccination. Vaccination programs use live oocysts which are administered using a hatchery spray or gel, a gel puck placed into hatchery box, or in-ovo dosing. These methods provide a prescribed amount of oocysts at an early age enabling immunity development to progress rapidly but still at a desired rate. A significant amount of immunological protection develops by 14 d of age, allowing birds to withstand a substantial challenge by 21 to 28 d of age. Coccidial vaccines are of two types; non-attenuated (not altered) and attenuated. All vaccines contain at least *E. acervulina*, *E. maxima*, and *E. tenella*. Non-attenuated vaccinated broilers' oocysts shedding starts with an early d 7 peak, a major peak at d 18-28, and then a decline. Attenuation of the coccidia causes the attenuated vaccinated broilers to generally start oocysts shedding approximately a day earlier, with a lower oocysts shedding peak, and extends longer than non-attenuated vaccinated broilers. Many other factors influence oocysts shedding including management, duration of drug program, breed, vaccine condition and application. To sustain good coccidiosis management all programs and influences need to be considered.

McDougald (3) stated that for an anticoccidial to be effective for the broiler industry it must have a broad spectrum of activity with a significant improvement in broiler performance. At the XIX World Poultry Congress (4), I stated that the criteria for selection of an anticoccidial to use in a broiler complex should be based in the following: cost, efficacy/ sensitivity, effect on acquired immunity, and toxicity.

Concentrating only on antibiotic free products, other than FDA approved anticoccidial drugs, there are very few products that meet these criteria. Several have demonstrated ability to reduce oocysts production; generally this has not translated into reduction in coccidial lesions or improved performance. From research that I have conducted none of these products have demonstrated the anticoccidial activity of the fully sensitive FDA approved drugs. However due to demand for antibiotic free birds, drug resistance, toxicity, or tissue residue issues these products are an option.

The two classes of products that I have tested that showed the most promise as an anticoccidial are the saponin or sapogenines (isolated from yucca or quillaja) and the essential oils (single EOS or blends). In a series of coccidia challenge studies, a yucca sponnin product reduced *E. acervulina*, *E. maxima*, *E. tenella*, *E, brunetti*, and *E. mitis* compared to non-medicated infected controls (5). The strongest activity was against *E. tenella*.

One of the issues with coccidia vaccination is that it is a live product which relies on immunity development thus birds are infected. Non-antiboitic products are often added to vaccination programs. Product, dose level, and timing of the addition are critical. In a coccidia challenge study with pure EO oregano with a low coccidia mixed species challenge demonstrated reduction in lesion scores. One of my criteria for a non-antibiotic product was to not interfere with coccidial immunity. A coccidia immunity study was conducted with this oregano product and coccidia vaccination. Oregano significantly reduced oocysts cycling and related coccidia immunity development. This information suggested that it was not appropriate to use this product in the starter feeds with a vaccine. Oregano product has been shown to work in the grower feed with vaccination after immunity has had a chance to develop. A proprietary mixture of organic minerals, yeast cell wall oligosaccharides and plant extracts has also been investigated (6). This product showed significant anticoccidial activity however not at a level to disrupt coccidial immunity development. Thus this product demonstrated advantageous benefits using continuously or only in the grower phase of a growout.

In discussing antibiotic free coccidiosis control we must also discuss enteric bacterial diseases. Coccidiosis coupled with associated enteric bacterial issues makes it the most costly disease facing the poultry industry. A survey of southeastern USA poultry veterinarians ranked disease issues with coccidiosis as number one and necrotic enteritis as number two (7). Intestinal damage by coccidiosis or other stressors (nutritional, environmental, etc.) enables *Clostridium prefringens* to proliferate and potentially cause necrotic enteritis. The most direct necrotic enteritis link is between *Clostridium prefringens* and coccidiosis with *E. maxima* being the leading cause (6). In certain circumstances with vaccination or drug failure, the infection will cause significant damage to the intestine which can lead to *Clostridium prefringens* proliferation and sub or clinical necrotic enteritis. The use of antibiotic

replacements now has a dual role reducing coccidiosis and necrotic enteritis.

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# EFFECT OF A SPECIFIC BLEND OF ESSENTIAL OILS AND OLEORESINS ON BOTH THE GROWTH AND IMMUNITY PARAMETERS OF BROILER

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## INTRODUCTION AND GOALS

Phytogenic feed additives (PFA) are plant-derived products used in animal feeding in order to improve performance of agricultural livestock. During the last decade, this class of feed additives has gained increasing interest, especially for use in swine and poultry, as can be derived from a significant increase in number of scientific publications since 2000 (3).

Therefore, a comparative trial was conducted to evaluate the effect of a blend of essential oils and oleoresins on broiler performance and immunity status e.g. antibodies (Ab) titration over a 42 d experiment. In fact, if natural essential oils and spices present well documented evidences of effectiveness of antibacterial (1) and digestive stimulant (2) activities, respectively, few data are available to their positive effect on both immune system establishment and efficiency.

## MATERIALS AND METHODS

**Housing and birds**. Three hundred sixty chickens (Ross-308, average initial live-weight of 41.6 ± 1.3 g) were randomly distributed into two groups with three replicates (3x60 birds) in each one. One day chickens were housed in the same building and were fed on a commercial feeding program based on three feeds: Starter (ME: 2820 Mcal/kg, CP: 22.1%), Grower (ME: 2942 Mcal/kg, CP: 20.2%) and Finisher (ME: 3130 Mcal/kg, CP: 18.1%).

***Vaccination program***. All chickens received the same vaccination program as follows:

**Newcastle disease (ND) vaccination**. The dose was divided into two parts (half dose by drinking water & other half dose by spraying system) using a ND vaccine (La Sota strain, Intervet SP product) at ages of 10, 20, and 28 d respectively.

**IBD (Gumboro) vaccination**. Two doses by drinking water at ages 7 and 14 d respectively using an intermediate Gumboro vaccine (StrainD78, Intervet SP product)

**Treatments**. The phytogenic feed additive (Oleobiotec®Poultry, Laboratoires PHODE - France) was supplemented (100 g/MT) to one of the treatments. This PFA is composed with blend of essential oil with standardized level of active volatile principles as phenolic substances (eugenol, carvacrol) and phenylpropanoids (cinnamaldehyde) and with a specific blend of oleoresins of spices that supply mainly vanilloids and capsaicinoids active molecules.

**Monitored parameters**. At the age of 1, 11, and 42 d, feed intake (n=360), weight gain (n=360), feed efficiency (n=360), mortality rate (n=360), and blood antibodies titration (n=16) (ELISA test) were completed.

**Statistical analysis**. The ANOVA procedure of SAS (SAS Institute, 2004) was achieved to compare the variance and means were separated using a Duncan's test and reported as significant at the $P<0.05$ level.

## RESULTS

The results showed significant increases of growth performance ($P<0.01$) as a direct and closely consequence of the significant improvement of the feed intake overall the trial period (Table #1). Although the feed intake is increased during the trial, the feed efficiency (FCR) is not negatively affected in the tested group (Table 1). Mortality rate is also significantly improved in the tested group (1.5 compared to 2.5% at 42 days for tested and control group, respectively).

Regarding the antibodies titers (Table 2), an interaction effect is observable: a non-significant decrease of ND titers is analyzed for the Newcastle vaccine response from tested birds whereas a significant improvement of Gumboro vaccine response appeared on the same birds compared to that of control ones.

## CONCLUSION AND DISCUSSION

The supplementation of broiler diet with the Oleobiotec Poultry (Laboratoires PHODE, France) feed additive stimulates significantly the total amount of feed intake increasingly ($P<0.05$) the growth performance of broilers. The experimental conditions characterized with very low sanitary pressure could explain the lack effect on feed efficiency that is normally observed on phytogenic supplemented animals (3). These controlled conditions could also explain the interaction on the immune response observed over the trial and depending of the analyzed titers. Therefore, by acting specifically on digestive tract (both intestinal secretion and microflora modifiers), the active substances phytogenic feed additive (Oleobiotec Poultry, - Laboratoires PHODE, France) have not improve the Newcastle preventive vaccination response whereas the better growth performance have probably improved the establishment and efficiency of the vaccination response against the Gumboro. Furthermore, the difference of vaccination program (ages and repetitions) could explain also the positive or no effect of the tested phytogenic additive on the vaccination responses.

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**Table 1.** Effect of the phytogenic additive on the zootechnical performances of broiler.

| Age | Treatments | Body weight (g) | Feed intake (g) | Growth performance (d/g) | Feed conversion (FCR) | Mortality % |
|---|---|---|---|---|---|---|
| 11 days | Control<br>Oleobiotec® | 208 ± 10.1 [a]<br>248 ± 11.0 [b] | 178. 7± 15.2 [a]<br>220.7 ± 13.8 [b] | 15.2 ± 2.6 [a]<br>18.8 ± 1.5 [b] | 0.86 ± 0.058 [a]<br>0.89 ± 0.117 [b] | 0.5 ± 0.13 [a]<br>0.0 ± 0.0 [b] |
| 25 days | Control<br>Oleobiotec® | 1023 ± 84 [a]<br>1108 ± 67 [b] | 1368.1 ± 81 [a]<br>1472.0 ± 83 [b] | 70.1 ± 4.8 [a]<br>76.2 ± 4.5 [b] | 1.34 ± 0.095<br>1.33 ± 0.088 | 0.0 ± 0.0<br>0.0 ± 0.0 |
| 35 days | Control<br>Oleobiotec® | 1867 ± 128 [a]<br>2073 ± 114 [b] | 2899.4 ± 103 [a]<br>3217.5 ± 89 [b] | 182.5 ± 22 [a]<br>203.2 ± 17 [b] | 1.55 ± 0.103<br>1.55 ± 0.098 | 1.5 ± 0.27 [a]<br>0.5 ± 0.10 [b] |
| 1-42 days | Control<br>Oleobiotec® | 2738 ± 94 [a]<br>2822 ± 92 [b] | 4696.1 ± 108 [a]<br>4820.2 ± 102 [b] | 64.3 ± 13 [a]<br>66.2 ± 9 [b] | 1.72 ± 0.098<br>1.71 ± 0.124 | 2.5 ± 0.15 [a]<br>1.5 ± 0.17 [b] |

[a – b] Means within columns with different superscripts differ significantly at ($P \leq 0.05$)
Non-significant differences within columns same letters.

**Table 2**. Effect of the phytogenic additive on the broilers' ELISA immune titers.

| Age | Treatments | ND | IBD (GUMBORO) |
|---|---|---|---|
| 1 day | For All | 7554 ± 38 | 6388 ± 286 |
| 11 days | Control<br>Oleobiotec® | 6026 ± 122 [a]<br>2988 ± 57 [c] | 2878 ± 69 [a]<br>4474 ± 85 [b] |
| 35 days | Control<br>Oleobiotec® | 3670 ± 64<br>2802 ± 41 | 839 ± 29<br>427 ± 20 |
| 42 days | Control<br>Oleobiotec® | 2990 ± 41<br>2188 ± 36 | 2437 ± 51 [a]<br>3099 ± 62 [b] |

# EFFECT OF A SPECIFIC BLEND OF ESSENTIAL OILS AND OLEORESINS OF SPICES AS AN ANTIBIOTIC GROWTH PROMOTER ALTERNATIVE ON BROILER PERFORMANCE

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## INTRODUCTION AND GOALS

There is currently considerable controversy regarding the use of antibiotics as growth promoters in poultry production, what has led to restriction or even a complete ban of these substances in some countries. Considering the simple removal of antibiotic growth promoters might have a negative economic impact, search for alternative additives has been incentivized.

Natural plant products, mainly essential oils, have been evaluated as possible feed additives for animal production, especially considering their in vitro antimicrobial activity (1). Moreover, an important propriety has also been observed on the gastrointestinal enzymatic activity, most likely improving nutrient digestibility (2).

Therefore, the main goal of this experiment is to evaluate the efficiency of a blend of essential oils and oleoresins on performance of broiler between 0 to 36 d compared to usual conditions of production based on antibiotic growth promoter (AGP).

## MATERIALS AND METHODS

**Housing and birds**. Two hundred eighty-eight chickens (Ross-308, average initial live-weight of 41.2 g) were randomly distributed into two groups with six replicates (6x24 birds) in each one.

One day chickens were housed in the same conditions (0.08 m² per bird, vaccinated at one d of age with Marek's and infectious bronchitis) were given *ad libitum* access to three feeds (ME: 2920 Mcal/kg, CP: 21.02%), Grower (ME: 3969 Mcal/kg, CP: 19.02%) and Finisher (ME: 3146 Mcal/kg, CP: 18.2%) and water for the whole trial period of 36 d.

**Protocol of contamination**. To recreate a similar commercial farms sanitary pressure, birds were placed on a new litter at beginning. In addition, the chickens in this trial were received Coxivac-B doses of vaccine at the time of their arrival (five times the normal dose administered). Thus, Coxivac-B vaccine was reconstituting in liquid form, and sprayed on the feed and water.

**Treatments**. Positive control feed is supplemented with Coyden (0-21 d), Salinomycin (22-36 d) and 55 ppm of BMD like growth factors (0-36 d of age).

In the tested group, only the BMD is substituted by a phytogenic feed additive (Oleobiotec®Poultry, Laboratoires PHODE - France) at 100 g/MT and all remains the same. This PFA is composed with blend of essential oil with standardized level of active volatile principles as phenolic substances (eugenol, carvacrol) and phenylpropanoid ones (cinnamaldehyde) and with a specific blend of oleoresins of spices that supply mainly vanilloids and capsaicinoids active molecules.

**Monitored parameters**. At the age of 7, 21, 28 and 36 da, feed intake (n=12), weight gain (n=12), feed efficiency (n=12), mortality rate (n=288) were completed.

**Statistical analysis**. Statistical analysis was carried out using the mixed procedure of SAS® software (SAS v. 9.3 Cary, N.C.). The effect of time and treatment on growth performance, feed consumption, and feed conversion data was analyzed with a repeated-measures linear model, with treatment as between-subject factor, time as within-subject factor, and pen nested within treatment as random factor.

## RESULTS

Both groups (Oleobiotec vs. positive control, BMD) present the same performance: mortality rate, growth performance and feed efficiency. The final live-weight (LW) at 36 d of age shows a significant improvement in the antibiotic free group (Oleobiotec): + 68.9 g/bird, i.e. +2.77%. The kinetic of mortality is slightly different between the two groups but the rate over the trial is exactly the same and remains low for the challenged conditions.

## CONCLUSION AND DISCUSSION

In this trial, the protocol of contamination helped to create condition close to commercial farm. Therefore, the phytogenic feed additive Oleobiotec Poultry formulated as a specific blend of essential oils and oleoresins could be proposed as an efficient alternative to substitute antibiotic growth promotor (BMD in this case). In economical point of view, a net profit expressed by kg of produced LW was also alculated over the trial and appeared to be in favor of the natural alternative : 0.558 vs 0.528 CA$ (+5.68 %) per kg of produced LW, for the Oleobiotec and positive control groups, respectively. Thus, today phytogenic alternatives can be proposed as efficient and profitable as usual antibiotic growth promoter strategy.


## ACKNOWLEDGMENT

We greatly thank the private experimental unit, Ferme des 3 chênes, where the trial was carried out under the supervision of Ghislaine Roch, agr. BSA, Ms Animal Nutrition during 2014 summer period.


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**Table 1.** Effect of the phytogenic additive on the zootechnical performances of broilers.

| Period | Groups | Body weight, g | ADG, g/d | Feed intake, kg/pen | Feed efficiency | Mortality rate, % |
|---|---|---|---|---|---|---|
| **0 - 7 days** | Control | 164.0 | 17.52 | 5.49 | 1.87 | 0.69 |
| | Oleobiotec® | 170.5 | 18.43 | 5.09 | 1.64 | 2,08 |
| **8 - 21 days** | Control | 904.3 | 52.88 | 24.76 | 1.40 | 1.40 |
| | Oleobiotec® | 929.3 | 54.20 | 24.28 | 1.33 | 0.71 |
| **22 - 28 days** | Control | 1553.2 | 91.47 | 28.67 | 1.89 | 1.42 |
| | Oleobiotec® | 1596.7 | 94.86 | 29.15 | 1.85 | 0 |
| **29 - 36 days** | Control | 2483.7[a] | 115.70 | 42.84 | 1.98 | 0 |
| | Oleobiotec® | 2552.6[b] | 119.06 | 43.55 | 1.94 | 0.71 |
| **0 - 36 days** | Control | [redacted] | 67.84 | 101.75 | 1.77 | 3.47 |
| | Oleobiotec® | | 69,75 | 102.07 | 1.72 | 3.47 |

[a – b] Means within columns with different superscripts differ significantly at ($P \leq 0.05$)
Non-significant differences within columns same letters.

# EVALUATION OF A HATCHERY ADMINISTERED LACTIC ACID BACTERIA PROBIOTIC PRODUCT ON EARLY BODY WEIGHT AND MORTALITY IN BROILER CHICKENS UNDER FIELD CONDITIONS

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## SUMMARY

Intestinal microbial colonization starts immediately after hatch. Under commercial conditions, maternal microflora transference is minimal and the chick's GI tract is exposed to a random bacterial population. Therefore, the application of a probiotic product after hatching could stimulate the GI tract development, protect against pathogenic bacteria, and consequently improve production parameters. In the present study, four field experiments were conducted to evaluate the impact of a neonatal probiotic product, FloraStart® (Pacific Vet Group USA Inc., Fayetteville AR 72701), on seven d body weight and mortality of broiler chickens. In experiments 1 and 2, a decrease in the seven d cumulative mortality was observed in the houses where chicks received probiotic in the hatchery. In experiments 2, 3, and 4, chicks sprayed with the probiotic product at the hatchery showed a significantly ($P < 0.05$) higher body weight at seven d of age compared to controls.

## INTRODUCTION

The chicken GI tract has a diverse microflora, containing more than 900 species of bacteria (1). In addition to biochemical functions, the microflora is responsible for digestion and subsequent nutrient absorption (2). One of the major influencers on the chicks' microbial colonization is the environment, since GI colonization starts immediately after hatch (2). Under commercial conditions, maternal microflora transference is minimal and the chick is exposed to uncontrolled bacterial colonization from machinery, boxes, trucks, personnel, and others (3).

Treatments with probiotic bacteria have been extensively studied under commercial conditions. The results from these studies showed that chicks respond best when probiotics are administered early in life (4, 5, 6, 7, 8, 9). Although application of probiotics during the brooding period may be indicated, the "gap" between hatching and placement may still be the crucial application period for achievement of full effect from probiotics. Colonization of a probiotic product immediately after hatching could stimulate GI tract development, protect against pathogenic bacteria colonization, support further microflora establishment, and provide a cascade of benefits through the chicks' lifetime, ultimately resulting in improved production parameters.

The neonatal probiotic product FloraStart was extensively studied under laboratorial conditions. The bacterial isolates present in this probiotic showed to successfully colonize neonatal chicks' GI tract and improve chicks' GI health during the critical first week of life (10). This manuscript's objective was to evaluate the impact of this neonatal probiotic on seven day mortality and body weight of broiler chickens under commercial conditions.

## MATERIALS AND METHODS

**Probiotic culture and application.** FloraStart is a poultry-derived, generally recognized as safe (GRAS) probiotic culture, consisting of two strains of lactic acid bacterial isolates: *Lactobacillus plantarum* TY036 and *Enterococcus faecium* MFF109. For application in commercial hatcheries, the powder form of the probiotic product was mixed with water following manufacturer's directions. Twenty mL of product solution was sprayed (coarse spray) per box of 100 chicks, allowing each chick to be exposed to an average dose of $10^7$ cfu of the bacterial isolates.

**Experimental design.** Subsequent to FloraStart spray application at the hatchery, each poultry company followed its standard procedures of transportation, placement, and brooding. In all trials, two groups were evaluated, a control group (not sprayed with probiotic) and FloraStart treated group. An experiment number was designated for each poultry company as follows.

**Experiment 1.** Chicks were placed during December 2013 and January 2014. Control groups consisted of 15 farms with a total number of 2,120,012 birds, and FloraStart groups consisted of

14 farms with a total number of 1,995,852 birds. Seven d mortality was evaluated.

**Experiment 2**. Chicks were placed during January and February 2014. Control groups consisted of 28 farms with a total number of 2,480,182 birds, and FloraStart groups consisted of 25 farms with a total number of 2,256,611 birds. Seven d mortality and body weight were evaluated.

**Experiment 3.** Chicks were placed in December 2013. Control groups consisted of 40 houses and FloraStart groups consisted of 42 houses. Seven d body weight was measured.

**Experiment 4.** Chicks were placed in August 2014. Control groups consisted of four houses and FloraStart groups consisted of four houses. Seven d body weight was measured.

**Statistical analysis.** Mortality cards were provided to authors for data analysis and calculated by averaging the seven d cumulative mortality rate on Microsoft Excel. Chicks' body weight was measured by weighing a group of 10 chicks per bucket, and 10 buckets per house (n=100 chicks/house). Body weight among groups was compared using the GLM procedure of SAS (11) with significance reported at $P < 0.05$. Means were further separated using Duncan's range test.

## RESULTS AND DISCUSSION

According to Creasey (12), seven d mortality is a good tool for measuring hatchery and brooding performance; likewise early mortality impacts final livability and performance. In two distinctive experiments, a decrease in the seven d mortality was observed in the houses where chicks received probiotic in the hatchery. In experiments 1 and 2, a decrease in mortality of 0.61% and 0.14% respectively, was recorded. Interestingly, in three distinctive field experiments, the probiotic product FloraStart showed improvements in body weight. In experiments 2, 3, and 4, chicks that were sprayed with the probiotic product at the hatchery showed a significantly ($P < 0.05$) higher body weight at seven d of age compared to control chicks (Table 1).

The mechanisms of action regarding decreased mortality and improved performance through the use of probiotics have not been elucidated. The most frequently described functions for the GI microflora are modulation of the immune system (13, 14), nutrient utilization, organic acids production, maintenance and renewal of intestinal epithelial cells and intestinal barrier, toxins breakdown, exclusion of pathogens, and improvement of excreta "quality" (15, 3). Changes in the GI microflora, which influences energy intake from feed, may cause variations on broilers' performance (16). Moreover, it has been reported that birds with different intestinal microflora presented differences in feed conversion rate after consuming the same feed (17).

In the present series of field experiments, the probiotic product FloraStart showed increased body weight and decreased overall mortality in the first seven days of life.

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**Table 1.** Body weight data from Experiments 2, 3, and 4 (7-day bucket weights groups of 10 chicks).

| **Experiments** | Control | FloraStart |
|---|---|---|
| Experiment 2 | 1382.65 ± 7.97 [b] | 1408.63 ± 8.07 [a] |
| Experiment 3 | 1434.95 ± 6.92 [b] | 1472.82 ± 5.56 [a] |
| Experiment 4 | 1540.28 ± 3.28 [b] | 1580.07 ± 2.91 [a] |

Values are expressed as mean ± standard error.

a,b Means with different superscripts within the same row, differ significantly ($P < 0.05$).

# LOW PATHOGENICITY AVIAN INFLUENZA (LPAI) H5N8 IN STANISLAUS COUNTY, CALIFORNIA

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## SUMMARY

During April of 2014, a commercial Japanese quail (*Coturnix japonica*) layer flock noted increased mortality and submitted five live and four dead quail to the Turlock branch of the California Animal Health and Food Safety Laboratory System. Polymerase chain reaction (PCR) tests were positive for Influenza A virus subtype H5. National Veterinary Services Laboratory isolated an LPAI H5N8 avian influenza virus from a pharyngeal swab sample.

The quail flock consisted of two houses; a layer and a brooder. The quail houses were environmentally enclosed houses. The affected quail layer house contained approximately 50,000 laying hens and 7,000 11 wk old males.

There were nine additional houses on this premises that housed Peking ducks for egg production. The duck houses were curtain sided buildings. Each house contained approximately 2,000 adult layers and 400 adult males of the same age group for a total of 21,600 ducks.

Management practices for the index premises included the processing of quail eggs, balut and quail carcasses which were sold wholesale to interstate and intrastate distributors and markets. Cull quail were frozen in a commercial freezer and sold to falconers. Daily mortality was collected from all of the company's premises and incinerated on the index premises. A hatchery located on the index premises was utilized for the production of duck balut, replacement quail and ducklings. Cull male ducklings were frozen and donated to a local wildlife rehabilitation center. During the identified infectious period no quail carcasses or frozen ducklings were distributed.

Depopulation of both species via euthanasia ($CO_2$) was complete within a week of diagnosis. Disposal of carcasses and poultry products (balut, hatching, and quail eggs) were via landfill. The litter was composted on site and disked into a fallow field. The houses were cleaned and disinfected and left empty for 30 d. Restocking was permitted following negative environmental tests. Flocks were tested 30 d after placement in each house.

There were two contact premises, a layer ranch and a brooder ranch, with Peking duck flocks owned by the same company as the index farm and considered to be epidemiologically linked but not affected. Spent ducks were transferred from the associated layer premises to a slaughter plant and processed for pet food. The contact premises were tested weekly for four wk and then released from quarantine.

In addition, in the 10 kilometer zone (16) commercial poultry premises were sampled twice at least 10 d apart and (5) squab producers were sampled once. In the three kilometer zone (12) Backyard premises were sampled once. The objective of the response was met which was to contain the H5N8 LPAI virus to the affected premises and prevent transmission to commercial or backyard poultry operations.

## RESULTS AND DISCUSSION

NVSL reported that the amino acid sequence at the hemagglutinin protein cleavage site was compatible with North American LPAI virus. The sequence was 99.1% similar to American green-winged teal isolate from Wisconsin in 2010. The H5 is 98.2% similar to an H5N5 virus isolated from a mallard in California in 2013 based on the amino acid sequence from the complete hemagglutinin gene. The neuraminidase gene is 95.7% similar to an isolate from a mallard in Alaska in 2006. Based on previous detection of LPAI viruses in wildlife and the presence of migratory birds on the index premises the most suspected source is wildlife. Supporting evidence includes no other detections in extensive surveillance conducted in backyards and commercial poultry ranches in the area combined with ongoing commercial and live bird market system surveillance.

Full genome sequencing was performed by Dr. Webby's group at St. Jude in Memphis, TN. The CA H5N8 was compared to the Korea H5N8 (HPAI) and no relationship was detected.

# AVIAN MYCOPLASMA CONTROL – CENTRAL FOR ANTIBIOITIC INDEPENDENT PRODUCTION

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## SUMMARY

The effects of avian mycoplasma infections are overt disease (often chronic), subclinical losses and antibiotic dependence. Routine administration of antibiotics in broilers at 18-22 d was developed to prevent chronic respiratory disease in vertically mycoplasma infected broilers. The timing of this administration is after all the birds are infected but before overt disease occurs. The routine administration of antibiotics to layers (and in some parts of the world breeders) every four to eight wk during lay is to similarly knock back mycoplasma on a regular basis. Mycoplasma infection freedom (MIF) was developed as a strategy to prevent these problems. It has one big problem which is that the birds in uninfected flocks are totally unprotected against infection. Live vaccines that are safe (low transmission) and efficacious (protecting against wild strain disease) can provide the additional benefit of increasing the resistance of flocks to mycoplasma wild strain infection. This is particularly important where neighbouring operations may not have the same priority on controlling both *M. gallisepticum* (MG) and *M. synoviae* (MS) and challenge is continuous. Having vaccines to protect against both MG and MS infection means that no antibiotics are needed. Other infections that routine antibiotics have controlled include *Brachyspira*, *Avibacterium*, and *Pasteurella* but these can be attacked with biosecurity, other interventions and vaccines as well and are not the universal problems that mycoplasma infections are. MIF is at the core of being able to farm poultry and egg layers in modern production systems without dependence on routine therapeutic antibiotic administration and perhaps with vaccination should be considered state of the art in high risk areas.

## INTRODUCTION

In chickens, MG and MS are chronic infections that have been causing big problems including disease, production inefficiencies and antibiotic dependence since the development of modern production systems. Although a lot of people have the opinion that MS infection has little impact in layer, or sometimes even breeder operations, this opinion has to be interpreted with an understanding of the antibiotic use in the organisation. Sometimes production people look at antibiotics in-production out whereas the veterinary view is antibiotics in- production out- therefore a bacterium is part of the problem.

Morrow (2014) described the rationale behind mycoplasma control being central to reducing antibiotic dependence in poultry industries. This paper will look at examples where antibiotic reduction has followed introduction of MS control by MSH vaccination and total mycoplasma control by MSH and ts-11 vaccination.

## MATERIALS AND METHODS

**Case studies of antibiotic reduction from introduction of live mycoplasma vaccination.** Case studies on the impact of the introduction of live mycoplasma vaccination on antibiotic usage were collected from individual supervising veterinarians (Table 1) in various parts of the world. Most were where MS associated disease was confirmed and antibiotics had been used for a long time with varying degrees of success in controlling clinical signs. The US study was where MSH vaccine was used under permit.

One study in Indonesia looked at a straight substitution from F strain vaccine and antibiotic to MG ts-11 and MS MSH vaccine with no antibiotic in a large poultry company with broiler breeders and layer breeders. The data presented concerns the broiler breeder side of the company and does not include benefits past DO broiler chicks or layer information. The DOC price here was chosen as 30 cents per chick while the feed price was $700/ton.

**Estimate of the total antibiotic usage in Europe combating MS infection.** Using previous data on the prevalence of MS infection in the Netherlands (1), a dose rate of 25 mg/kg and treatment regimens typical of European production systems, an estimate of the total amount of antibiotic used against MS in the EU was made. No estimate was made for antibiotic use benefits in broiler progeny production.

## RESULTS AND DISCUSSION

**Indonesian case study.** A Cobb PS programme with 800K PS was having problems with respiratory disease despite using extensive

antibiotics and F strain vaccination. No diagnostics were undertaken. The results were dramatic with a cure of clinical signs (perhaps they were having problem with tylosin resistant mycoplasma) (4).

**An estimate of the amount of antibiotic used to combat MS infection in Europe**. The accuracy of this estimate is hard to assess but it is a starting point. Over the years of use of these vaccines in Australia the dependence of the poultry industries on routine antibiotics given at therapeutic levels has markedly dropped.

Anecdotally the introduction of MSH in Iran with extensive use in broiler breeders was associated with one million dollars, wholesale price, of tylosin (generic) expiring in storage.

## CONCLUSION

The economic benefits of mycoplasma control, especially mycoplasma control in layers needs to be revisited. The effects reported on FCR in the conversion of feed to eggs (5) or saleable chicks need to be confirmed. Indeed studies on this effect from MG infection also are warranted. The reduction in feed costs pays for the vaccination in real time – each feed bill – independent of the market prices realized for products.

With the push for a reduction in antibiotic dependence in poultry production systems, live mycoplasma vaccines need to be considered as a tool. These examples show that live mycoplasma vaccination of breeders and layers (in areas of significant challenge) can be expected to reduce the dependence of these industries and perhaps broiler production as well on the dependence of routine antibiotic treatments. The secret is to stop/review use of antibiotics after the introduction of vaccination (and not to combine the vaccination with killed mycoplasma vaccines). MIF needs to be updated to include considering vaccinated breeder flocks as being mycoplasma free for regulatory purposes or a new category (vaccinated).

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**Table 1**. Case studies with MSH.

| Country (when) | Segment/size | Problem | Antibiotic reduction |
|---|---|---|---|
| Germany 2008-9 | Broiler breeder 250K breeders | MS infection post transfer to rearing site | Reduction of 34 to 73% of antibiotic usage in breeders and 13.5% in broilers post MSH introduction |
| South Africa 2008-9 | Layer rearing 1 M layers | Infectious synovitis with vertical infection of layers | 100% reduction in antibiotic (aivolsin/doxy/tiamulin) use post MSH introduction |
| Mexico | Broiler breeders and broilers | Airsacculitis in broiler breeders and broilers | Elimination of enrofloxacin treatment of broilers and tylosin treatment of breeders after MSH introduction into broiler breeders. |
| USA 2009-2010 | Broiler breeders 82K breeders | MS infection and airsacculitis and vertical transmission to broilers | Elimination of CTC and tylosin use and better control of field challenge by MS after MSH introduction. |

These studies were compiled for submission to the EMA to support the registration of MSH in 2011.

**Table 2.**

| **Parameter** | **Before: on F-Strain Upto 2012** | **After: on TS-11/MS-H From 2012** | **Cost savings on new programme (USD)** |
|---|---|---|---|
| Respiratory health | Clinical respiratory disease | No respiratory signs in vaccinated birds | |
| Antibiotic usage | Routine – every six wk in feed Tylosin<br><br>Placement Medication with Tilmicosin and Enrofloxacin | None<br><br>Placement Medication with Tilmicosin to flush before vaccination | + $85, 000/year |
| Saleable chicks to 68 wk | 140 chicks | 162 chicks | +$3.50<br>Avg chick price: $0.30 |
| Feed required to produce one chick<br>(from production onwards) | 325g/chick | 285g/chick | +$0.30/ chick |
| Hatchability | 89% peak with average 84.2% | 92% peak with average 89% | |
| Airsacculitis in pipped embryos. | >35% | Up to 20% but good hatchability | |
| Chick quality | | Improved | |
| Cumulative Mortality | 10.2% (Female)<br>19.1% (Male) | 8.3% (Female)<br>16.5% (Male) | Less peritonitis in breeders observed in PMs. |

**Table 3.** Antibiotic usage against MS in Europe- 2011 estimate.

| **Sector** | **Number** | **MS incidence*** | **Antibiotics** |
|---|---|---|---|
| Broiler parent stock (and GP) | 14 M | 35% | 5.3 tons |
| Rearing from infected GP stock | 4.4 M | 100% | 1.2 tons |
| Broilers | | | No estimate |
| Layer | 264.8 M | 73% | 59.6 tons |
| Layer rearing from infected parent stock | 99.5 M | 100% | 12.4 tons |
| | | From Feberwee | |
| **Total antibiotic** | | | 78.5 tons |

# DIATOMACEOUS EARTH AS A NOVEL ADJUVANT IN POULTRY VACCINES

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## SUMMARY

We tested the capability of diatomaceous earth (DE) as a potential adjuvant for a killed Newcastle disease virus (kNDV) vaccine in chickens. At 18 d of embryonation, eggs were inoculated with one of the following experimental vaccines: DE+PBS, DE+kNDV, incomplete Freund's adjuvant + kNDV (IFA+kNDV), PBS+kNDV, Aluminum hydroxide gel + kNDV (Alum+kNDV), or PBS. The final concentration of kNDV per inoculum was $10^5$ $EID_{50}$/100uL. Hatchability, chick quality, and body weight were assessed at hatch. At 21 and 29 d of age, each group of chicks was boosted subcutaneously with 200uL of the respective experimental vaccine. Serum was tested for antibody titers at 7, 14, 21, 29, 36, 39, and 44 d of age. No significant differences were detected among groups in hatchability, chick quality, and body weights. Positive antibody titers were detected in DE+kNDV, IFA+kNDV, and Alum+kNDV groups at 36 d and thereafter. The IFA+kNDV vaccinated chickens showed statistically higher NDV antibody titers compared with DE and Alum+kNDV vaccinated chickens at 44 d of age. No differences in antibody response to NDV were detected between DE and Alum kNDV vaccinated chickens throughout the whole experiment. Our results experimentally suggest that DE could serve as a potential adjuvant for vaccines against diseases in poultry.

## INTRODUCTION

Rapid advances in the poultry industry require new vaccine formulations and strategies in order to protect flocks against infectious diseases. Such new vaccines should be stable, safe, and inexpensive to produce. Inactivated vaccines are commonly used in livestock and are considered the safest vaccines even though, their cost is sometimes prohibitive due to the use of adjuvants (5). Adjuvants increase the vaccine immunogenicity by serving as a depot for antigens in tissues, and activating the innate immune response, particularly macrophages to increase antigen presentation (8).

DE is natural siliceous sediment of fossilized remains of diatoms, which are single cell eukaryotic microalgae encountered in all water habitats (2). Due to structural, mechanical, chemical, and optical features of DE silica, DE can be utilized as drug delivery carriers (1, 9). In addition, silica has been reported to have higher colloid stability than Alum and to act as an adjuvant (4). In this experiment we tested DE as a candidate adjuvant in kNDV vaccines in chickens.

## MATERIALS AND METHODS

Inactivated vaccines against NDV were prepared using a live attenuated NDV LaSota vaccine strain. NDV was inactivated using 0.2% formaldehyde and magnetic stirring for six h at 25° C. The titer of the virus before inactivation was 2 X $10^6$ $EID_{50}$/mL. Specific pathogen free (SPF) embryonated eggs (192) were randomly divided into six groups at d 18 of embryonation. Each group of 32 eggs was inoculated with one of the experimental vaccines via the amniotic sac route (100uL/egg). Group 1 was inoculated with PBS containing 1 mg DE per inoculum. Eggs in groups 2, 3, 4, and 5 were inoculated with 1 mg DE + kNDV, IFA+kNDV, PBS+kNDV, and Alum+kNDV, respectively. The final kNDV titer received by each egg was 1 X $10^5$ $EID_{50}$. Finally, Group 6 was inoculated with PBS. Subcutaneous boosters of each experimental vaccine (2 X $10^5$ $EID_{50}$/200uL) were given at 21 and 29 d of age in the base of the neck.

Hatchability, body weight, morbidity, mortality, and chick quality were recorded. Chick quality measurements included navel score (7), chick length (3), leg condition, and residual yolk weight. Blood was collected from all birds via wing vein puncture at 7, 14, 21, 29, 36, 39, and 44 d of age. Serum was extracted and tested for antibodies using a commercial NDV enzyme-linked immunosorbent assay (ELISA) (IDEXX, Westbrook, Maine). Data

was statistically analyzed using Prism Graphpad 5 (San Diego, CA).

## RESULTS AND DISCUSSION

Minor variations were detected among groups in hatchability (Table 1). It has been reported that the best estimators of chick quality are chick weight and residual yolk percentage (6). In this experiment minor variations were detected in residual yolk percentage while chick length was uniform among groups. No statistically significant differences were detected in the previously mentioned parameters (Table 1). Additionally, DE+NDV group had the healthiest navels with a score of 1.5, even though, no significant differences were detected. Live BW varied slightly among groups (Table 1). We did not record any morbidity or mortality during the whole experiment.

Surprisingly, no NDV antibody titers were detected after *in ovo* vaccination and up to 29 d of age (Fig. 1). This result may be suggesting that the used kNDV titer was not sufficient to generate a measurable humoral immune response when inoculated *in ovo*. After the two subcutaneous boosters only DE+kNDV, IFA+kNDV, and Alum+kNDV groups displayed NDV antibody titers starting at d 36 of age. No statistical differences were detected among the three groups. The IFA+kNDV vaccinated chickens showed statistically higher NDV antibody titers compared with DE and Alum+kNDV at 44 d of age. No statistical differences in antibody response to NDV were detected between DE and Alum throughout the whole experiment (Fig. 1).

In conclusion, our results experimentally suggest that DE could serve as a potential adjuvant for vaccines against diseases in poultry. DE is a safe and non-toxic adjuvant for *in ovo* and subcutaneous vaccination of chickens. More research needs to be done in order to assess the real adjuvant capabilities of DE.

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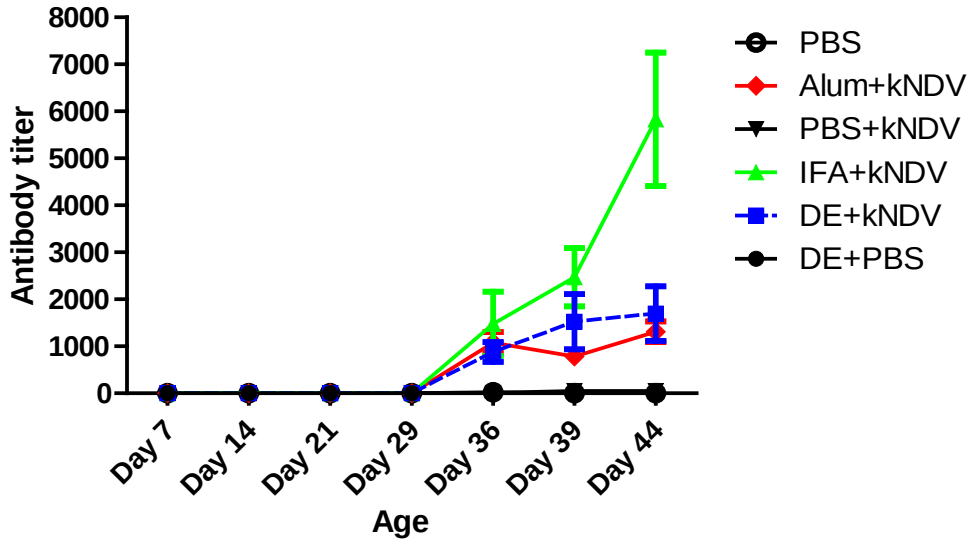
(This information will be published in the *Avian Diseases*.)

**Table 1.** Effect of different *in ovo* vaccines on hatchability, body weight and chick quality.

| Groups | Hatchability (%) | Body weight (g) | | | | Navel score* | Chick length (cm) | Residual Yolk % |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 7 | Day 14 | Day 21 | | | |
| **DE+PBS** | 100 | 44.4 | 77.2 | 139.1 | 224.8 | 1.75 | 17.2 | 9.7 |
| **DE+kNDV** | 96.97 | 44.6 | 77.2 | 142.2 | 230.5 | 1.5 | 17.1 | 9.67 |
| **IFA+kNDV** | 93.75 | 44.6 | 76.5 | 141 | 226.3 | 1.86 | 17.3 | 7.83 |
| **PBS+kNDV** | 90.63 | 44.4 | 79.3 | 141.2 | 223.8 | 1.52 | 17.2 | 7.34 |
| **Alum+kNDV** | 100 | 43.1 | 75.8 | 136 | 215 | 1.63 | 17.1 | 8.73 |
| **PBS** | 96.67 | 44.5 | 76.5 | 141.3 | 231.4 | 1.65 | 17.2 | 7.25 |

*Navel score: 1 for a clean and closed navel; 2 for a discolored button with open navel < 2mm, or black string; and 3 for a black button with open navel > 2 mm.

**Figure 1.** Mean serum NDV antibody titers at different chicken ages measured by ELISA. Error bar represents standard error. Priming was given at 18 days of embryonation, boosters at 21 and 29 days of age.



# ANTIMICROBIAL USE ON BROILER CHICKEN FARMS IN ONTARIO, CANADA

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## ABSTRACT

Knowledge of antimicrobial use in poultry production in Canada is limited (1). The study objectives were to describe the types and quantities of antimicrobials used on broiler chicken farms in Ontario, Canada and to compare two metrics for ranking antimicrobial use: mg of active ingredient/kg-bird produced and Animal Daily Dose (ADD)/kg-bird produced. Questionnaire-derived data from 68 conventional, 34 antimicrobial-free, and seven organic broiler flocks raised between July 2010 and April 2012 were analyzed. Two antimicrobial-free flocks were reclassified as conventionally raised due to disease treatment using antimicrobials, representing a 5.5% failure rate in this sample to maintain antimicrobial-free status. Sixty seven of 68 conventional flocks used at least one of 17 antimicrobials in-feed, and 27% of flocks (n=66) used an antimicrobial in-water. Eighteen percent of flocks (n=68) reported extra-label drug use. Considering all feeding phases together, the most commonly used antimicrobials measured as a percentage of flocks reporting use in at least one feeding phase were; bacitracin (64.7%), narasin-nicarbazin (45.6%), salinomycin (44.1%), followed by arsenical (41.2%) and monensin (38.2%). For the overall growing period, the five highest ranked antimicrobials based on mg of active ingredient/kg-bird produced were bacitracin, monensin, salinomycin, arsenical, and narasin. The five highest ranked antimicrobials based on ADD/kg-bird produced were bacitracin, monensin, salinomycin, virginiamycin, and arsenical. No antimicrobials classified under Health Canada's Veterinary Drugs Directorate highest category of importance to human medicine were administered; however, various antimicrobials indicated as very important were used. This study provides a profile of antimicrobial use on broiler chicken farms in Ontario, Canada and a baseline for monitoring antimicrobial use trends. The relative ranking of antimicrobials used in-feed varied depending on the metric chosen suggesting the importance of careful consideration of the appropriate metric depending on study objectives.

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# PREVALENCE, RISK FACTORS, AND PROFILES OF RESISTANT *SALMONELLA* ISOLATES OBTAINED FROM ONTARIO BROILER CHICKEN FARMS AT CHICK PLACEMENT AND PRE-HARVEST

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## ABSTRACT

There is rising concern that the continued use of antimicrobials in broiler chicken production leads to the contamination of poultry products with resistant *Salmonella* spp. followed by the development of resistant salmonellosis infections in humans (1). The study objective was to determine the prevalence and risk factors for resistance in *Salmonella* isolates obtained from broiler chicken flocks, with an emphasis of identifying differences between production types. Seventy-four conventional, thirty-four antimicrobial-free, and seven organic flocks distributed throughout Ontario, Canada were enrolled. For each flock, two pooled environmental dust samples were collected prior to chick arrival, and six pooled swabs of meconium on chick pads representing a hatchery-level sample were collected upon chick arrival (*chick placement*). Various sample types were collected within seven d of processing (*pre-harvest*). Standard techniques were used for *Salmonella* isolation and susceptibility testing to a panel of antimicrobials important to human medicine. A questionnaire administered to the producer gathered hatchery and barn-level information. Regardless of sampling stage or sample type, resistance was most commonly identified to the betalactam antimicrobials: ampicillin, amoxicillin-clavulanic acid, cefoxitin, ceftiofur, and ceftriaxone; in addition to other antimicrobials streptomycin and tetracycline. Resistance patterns varied among serotypes. The diversity of multidrug resistance patterns was greatest among *Salmonella* Kentucky isolates at both sampling stages. Multivariable generalized estimation equation fitted models were created for chick pad samples and environmental samples separately at chick placement, as well as for all sample types combined at pre-harvest (all serotypes combined, as well as serotypes considered separately). The only difference between production types was that *Salmonella* isolates (all serotypes) originating from environmental samples collected from antimicrobial-free flocks at chick placement had significantly lower odds of resistance to streptomycin (OR = 0.1; $p$ = 0.039) compared to isolates originating from conventional flocks, controlling for flock-level clustering. The Ontario hatchery company from which the chicks originated, resistance at chick placement, and the use of coveralls or designated barn clothing were often risk factors for resistance to the antimicrobials modeled. Results suggest that production system type has little impact on the prevalence of resistance, likely as a result of the transmission and persistence of resistance from higher in the production chain.

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# RISK FACTORS FOR ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* ISOLATES FROM ONTARIO BROILER CHICKEN FLOCKS AT CHICK PLACEMENT: A COMPARISON OF THREE PRODUCTION SYSTEM TYPES

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## ABSTRACT

Antimicrobial use in broiler chickens has been identified as a risk factor for the development of antimicrobial resistance in pathogens that can be transferred from chicken products to humans (2). It is a common perception that products raised and marketed as organic or raised without the use of antibiotics/antibiotic-free are of a higher microbial quality and that the purchase of these products reduces the risks associated with antimicrobial resistance and drug residues (1). However, data are lacking in Ontario that highlight the impact of these alternative production types on the level of antimicrobial resistance in broiler chickens. The study objective was to determine prevalence, antimicrobial resistance profiles, and risk factors for resistance in *Escherichia coli* isolates obtained at chick placement from broiler chicken farms, placing an emphasis on any differences which exist between production types. Seventy-four conventional, thirty-four antimicrobial-free, and seven organic flocks were sampled throughout July 2010 to April 2012. For each flock, two pooled environmental dust samples were collected prior to chick arrival, and six pooled swabs of meconium on chick pads representing a hatchery-level sample were collected upon chick arrival. A questionnaire administered to the producer gathered hatchery and barn-level information. Samples were submitted for *E. coli* isolation and susceptibility testing to a panel of antimicrobials important to human medicine using standard techniques. No resistance of *E. coli* obtained from chick pad samples to ciprofloxacin or from environmental samples to ciprofloxacin and azithromycin was identified. Logistic regression models, with random effects for the flock and producer levels, were built separately for chick pad and environmental samples and used to identify risk factors for resistance to each antimicrobial. The only difference between production types was that isolates from environmental samples collected from antimicrobial-free flocks had decreased resistance to trimethoprim-sulfa vs. conventional flocks (OR = 0.1; $p$ = 0.031). Based on models utilizing isolates from chick pad samples, hatchery-level risk factors for resistance included the use of infectious bursal disease vaccines (several antimicrobials), the use of Marek's disease vaccines (tetracycline only), and low age of the breeder flock(s) (tetracycline only); whereas protective factors included some Ontario hatchery companies from which the chicks originated (several antimicrobials), and the use of coccidiosis vaccines (trimethoprim-sulfa only). Based on models utilizing isolates from environmental samples, barn-level protective factors for resistance included all-in-all-out practices (several antimicrobials), and disinfecting the barn before chick placement (streptomycin only). This study has shown that, at chick placement, few differences exist between conventional and alternative broiler chicken production types with respect to the prevalence and antimicrobial resistance profiles of generic *E. coli*, and that risk factors for resistance can be attributed to both hatchery and barn-level factors and vary depending on antimicrobial agent.

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# SEROTYPING AND VIRULENCE GENE DETECTION IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM PROCESSING PLANT, SUPERMARKET, AND PUBLIC MARKET IN MEXICO

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## INTRODUCTION

In Mexico, 20% of the total broiler meat produced is sold in public market, whereas 15% in supermarket (13). During transportation of chicken carcasses, product handling is often inadequate, plus they do not meet the necessary hygiene measures. The cold chain is not preserved during the transportation and sale, which contributes to deteriorating microbiological quality, because there is no regulation that specifies the necessary hygiene conditions for manipulation and measures to preserve the product. In consequence, risk of foodborne illness in humans increases when a high rate of pathogenic microorganisms are present during birds rearing, processing, transportation and marketing.

Diarrheagenic *E. coli* strains are the leading cause of gastroenteritis in children in developing countries and are responsible for 30-40% of all cases of diarrhea in children (10, 11). While most meat products should be properly cooked before consumption, *E. coli* in meat can represent a risk for consumers, since cross-contamination with hands, vegetables, utensils or surfaces can occur if an inappropriate handling occurred before consumption (5). Several clones of *E. coli* have acquired specific virulence attributes, which give them the ability to adapt to new niches, allowing cause a wide spectrum of diseases. These virulence factors has led to the classification of specific pathotypes that are able of causing intestinal and extraintestinal diseases in humans; such as diarrhea, urinary tract infections, neonatal sepsis and meningitis (1, 2, 4). For those that cause digestive diseases, at least five groups based on their virulence traits have been described (6, 7), enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and enterohemorrhagic (EHEC ) (7).

Chicken meat is the product of animal origin with a higher demand in Mexico; for this reason it is necessary to evaluate the microbiological quality of chicken carcasses. To define the epidemiological importance of chicken in the transmission of pathogens, we use *E. coli* as model, since this is an important pathogen that affects both birds and humans. The aim of this study was to determine serotypes and virulence genes in *E. coli* strains isolated from chicken carcasses in processing plant, public markets, and supermarkets, because in our country there is no information regarding the health status of the chicken and presence of diarrheagenic groups of *E. coli* during processing and marketing of broilers.

## MATERIAL AND METHODS

Ten broiler carcasses were sampled at TIF processing plant located in the center region of Mexico, ten were purchased on three public markets and ten in three supermarkets in the surrounding area of the processing plant. All samples were kept refrigerated for later evaluation in the laboratory. To determine the microbiological quality of carcasses, rinsing technique were used. Each eviscerated carcass was placed in a sterile plastic bag, where 100 mL of sterile phosphate buffer solution (PBS) 1x were added, carcass washing was performed for 1 min with PBS covering internal and external surfaces; then 1 mL of washing were taken to perform four tenfold serial dilutions; 1 mL of each dilution was seeded in RIDA COUNT microplate for total count and total coliforms. The microplates were incubated 48 h at 37°C for further counting. For each sample five aliquots of PBS (1 mL), each sample was seeded into 10 mL of nutrient broth and incubated at 37°C overnight, then, each broth was streaked onto MacConkey Agar. For each sample a colony for identification was selected by biochemical tests (urea, SIM, citrate, TSI, LIA). A total of 150 strains of *E. coli* (five for each chicken carcass) were isolated. Serological identification was performed by the technique used in the laboratory of Bacteriology Department of Public Health, Faculty of Medicine, UNAM according to Orskov and Orskov (9). For the

determination of virulence genes nine specific sequences by PCR analysis for the virulence factors genes related to the five diarrheagenic *E. coli* groups were used as describe previously (3, 6, 8).

## RESULTS AND DISCUSSION

Public market carcasses showed the highest values of both, total count and coliforms (6.829 and 5.999 CFU/mL log 10, respectively), followed by supermarket carcasses, and the lowest counts were found in processing plant carcasses (5.604 and 4.817 CFU/mL log 10, respectively); processing plant and supermarkets were not significantly different (P> 0.05). The use of aerobic mesophilic count and total coliforms are a tool to control the quality of animal products and a high count indicates poor hygiene and inadequate storage temperature. Therefore, the fact that public markets showed the highest aerobic mesophilic and total coliforms counts, highlight the deficiencies that occur in these places, such as excessive handling, lack of refrigeration and contact with the surrounding environment. This fact suggests that these carcasses may become contaminated by pathogenic microorganisms, since they are mesophilic. Furthermore, the shelf life of a product is determined by the initial bacterial load, storage temperature and the atmosphere around the product; for this reason, the high counts we found decrease shelf life of the carcasses. *E. coli* was present in all sampled carcasses, which is not unusual because this microorganism is part of the intestinal biota of humans and animals and, as already mentioned, their presence in food is considered an indicator of fecal contamination. The microbiological quality of carcasses is a reflection of the microbial load of live poultry and hygiene on the processing plants. Birds that are incorporated into the slaughter can be contaminated with microorganisms carried in the gut, skin and feathers, including enteric bacteria, as well as environmental ones.

One-hundred-and-fifty strains of *E. coli* belonging to 48 serogroups were identified, being O8 (16.5%), O7 (13.5%), O48 (13.5%), ONT (13.5%), O103 (12%), O11 (12%), O15 (12%) and O6 (10.5%) the most frequent. Rosario *et al*.(12) identified serogroups O8, O103, O15, O84 in samples from birds with yolk sac infection in a commercial farm, whereas Morales *et al*., isolated O8, O7, O15 serogroups from backyard birds. These serogroups match with those reported here which suggests that some of the strains found in this study could have originated from farm. Thirty different serogroups were isolated from public markets, whereas only 19 (in each place) were identified in both, supermarkets and processing plant. This information is consistent with the fact that in public markets hygiene is poor, leading to an increase in the amount of serogroups in these places and, in consequence, an increase in the risk to consumer health. It is noteworthy that serogroup O157 were isolated in public markets samples, which according to reports, is not common in birds, therefore this fact suggest that this strains could have human origin. The strains of serotype O157: H7 have been described within the EHEC group, which has been implicated in outbreaks of hemorrhagic colitis, and even deaths from HUS. However the strains O157 isolated was not motile. Moreover, 12 out of the 19 serogroups of *E. coli* isolated from processing plant were also found in either public market or supermarket samples, this could indicate that contamination is present from farm or processing plant. In total 78 different serotypes were obtained, the most frequent were O103: H21 (n=8), O11: H6 (n=8) and O7: H4 (n=7). Some of them were isolated more than once from the same sample, which indicates that these strains could be colonizing birds.

Five percent (8 strains) were positive for *stx*2 gene, six from supermarkets and two from public markets. On the other hand, six strains possessed *eae* gene, five of them from public markets and only one from the processing plant. In summary, only 14 out of the 150 isolates were positive for virulence genes (*stx*2 or *eae*). In consequence, these findings could indicate that most of the *E. coli* isolated from chicken carcasses are commensal strains from the digestive tract of birds and the result of fecal contamination.

In conclusion, the results shows a wide variety of serotypes isolated in the three points of sampling, however we found the larger group in public market, possible related to a contamination that occur after the carcasses left the processing plant, that could be related with a inappropriate handling of the product. Even more, only one strain isolated from processing plant was positive for virulence genes, whereas the other 13 positive were isolated in public market and supermarket. These results, could indicate that despite contamination of the broiler carcasses during and before processing can occur, those strains that contaminate carcasses during transportation, handling, and sale, could represent even a higher risk for humans since most of the virulence genes were isolated from retail stores, actually these strains could have a human origin, since they were not found in the processing plant.

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# INCREASING STANDARDIZATION IN REAL-TIME PCR DIAGNOSTICS WITH A MODULAR SYSTEM AND ITS BENEFIT TO LABORATORY THROUGHPUT

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## SUMMARY

IDEXX RealPCR™ real-time PCR reagents aim to provide a new level of standardization to PCR diagnostics by offering reagents in a modular system. The components of the DNA and RNA modular systems are shared over the entire platform, making it possible to run any pathogen-specific detection mix with a standard master mix and a single positive control. Moreover, the RealPCR modular system maintains a single PCR cycling program for all DNA and RNA targets, enabling reactions for multiple targets to be performed simultaneously.

## INTRODUCTION

Real-time polymerase chain reaction (PCR) has revolutionized diagnostic testing over the past decade, and is currently used in a variety of diagnostic applications worldwide. While real-time PCR has offered significant advantages over end-point PCR, commercial real-time PCR assays still use a set of reagents, or "kit", designed for testing a precise number of samples for a specific target(s). This approach often requires a separate testing protocol for each target, increasing time to results and hands-on time for laboratories. IDEXX RealPCR reagents are designed to work as a modular system. Thus, the RealPCR DNA or RNA master mixes may be used with any of the respective DNA or RNA RealPCR detection mixes. Additionally, a single pooled positive control works as a PC for any RealPCR assay. To increase testing efficiency, all RealPCR reagent sets are designed to utilize a single cycling protocol allowing DNA and RNA assays to be run side-by-side. Finally, when suitable, the IDEXX RealPCR reagents utilize an internal sample control (ISC) for the detection of endogenous host DNA or RNA. The ISC performs the same function as an internal positive control (IPC) but also confirms sample quality and collection while not requiring a separate spiking step. The RealPCR Quality Control (QC) reagents provide additional tools for environmental contamination monitoring programs. The RealPCR QC Signature Detection Mix specifically identifies positive control contamination in laboratories, while the RealPCR QC Pan Bacterial Detection Mix is useful as a positive control when running environmental control samples. As an early offering to this modular system, multiplexed, real-time PCR reagents have been developed for the simultaneous detection and differentiation of DNA from two avian pathogens, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS).

## MATERIAL AND METHODS

**Sample DNA extraction.** DNA was extracted from avian tracheal swabs using either a commercial extraction kit (Qiagen) or with a buffer-boiling method as follows. Swabs were eluted either individually or as pools (n = 5) in 300 µL increments of PBS and the resulting material heated to 95°C for 10 min followed by incubation on ice for 15 min before using directly in PCR reactions.

**Multiplex real-time PCR design**. Sequences unique to either MG or MS were previously identified (1). Primers and probes were designed to amplify and detect the presence of these targets sequences in a multiplex format (2), while also conforming to the RealPCR cycling protocol outlined below in Table 1. For the ISC, a sequence conserved between chickens and turkeys but unique from all other sequences currently residing in the NCBI database (http://blast.ncbi.nlm.nih.gov/) was identified and primers and a probe were designed to amplify and detect the presence of this sequence.

**Real-time PCR standard curves and conditions.** Quantified synthetic DNA representing target sequences unique to either MG, MS or ISC was used. Sequences were individually diluted in 10-fold increments to obtain 10 copies per 5 µL. Amplification reactions were performed in a total volume of 25 µL, with all samples in triplicate. Each reaction contained 10 µL of both RealPCR Master Mix and RealPCR MG/MS detection mix, and 5 µL of sample. Reactions were incubated at 50°C for 15 min, 95°C for one min followed by 45

cycles of 95°C for 15 s and 60°C for 30 s with fluorescent signals taken at the end of each extension step in the FAM, HEX, ROX, and Cy5 channels (Table 1).

## RESULTS

To determine the efficiency of the MG/MS multiplex test, primers and probes for the amplification of MG, MS and ISC were combined into a single detection mix. Diluted synthetic MG or MS target was tested over an 8-log range starting with 10 copies of target per reaction up to $10^8$ copies per reaction, in triplicate, to generate a standard curve (Fig. 1). Both MG and MS reactions exhibit efficiencies of 99.9% with correlation coefficients of 0.998 or greater. Additionally, the ISC reaction has an efficiency of 96.4% with a correlation coefficient of 0.989 (data not shown). This testing demonstrates high efficiency for all three targets and confirms the reagents perform as expected using the RealPCR cycling conditions.

The RealPCR MG/MS reagents detected ISC in 100% of all samples tested, regardless of the purification method (data not shown), demonstrating the effectiveness and robustness of the design.

## CONCLUSION

The IDEXX RealPCR modular system allows the user to include different assays in a single PCR plate, overcoming the limitation of low volume sample sets delaying a PCR run for the laboratory, making it efficient to include low volume PCR assays into the same run as a high volume assay. Smaller laboratories might also find PCR testing more feasible with the use of this system, since total sample numbers for a PCR run are not assay-dependent. Moreover, the use of modular components makes the introduction of new assays easier. Following the IDEXX RealPCR modular system, the MG and MS real-time PCR multiplex reagents demonstrates high efficiency with robust performance with different sample types. We believe the shared reagents and standardized testing of the IDEXX RealPCR modular system will greatly increase efficiency, while decreasing real-time PCR results turnaround time for many diagnostic laboratories.

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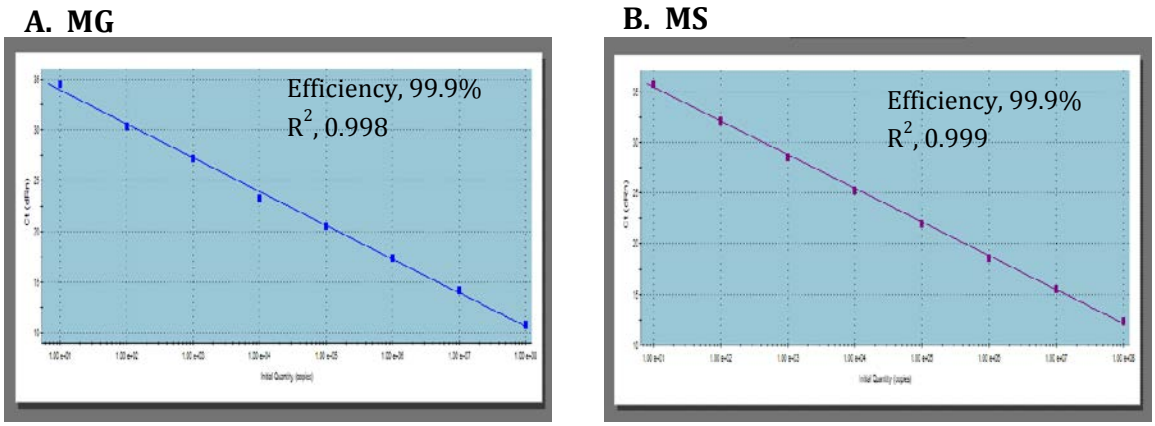
**Table 1.** IDEXX RealPCR standard cycling protocol.

| | | | |
|---|---|---|---|
| Step 1 | 50C<br>95C | 15 min.<br>1 min. | 1 cycle |
| Step 2 | 95C<br>60C | 15 sec.<br>30 sec. | 45 cycles |

**Figure 1.** Standard curves with efficiencies and correlation coefficients for the RealPCR MG/MS multiplex reagents. (A) MG results using FAM channel. (B) MS results using Cy5 channel.



# ASSESSMENT OF CHANGES IN SENSITIVITY OF COCCIDIA TO NICARBAZIN

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## INTRODUCTION

Nicarbazin (Nicarb®) is a synthetic anticoccidial that has been successfully used to prevent coccidiosis in broiler chickens since 1955 (1). The nicarbazin premix contains 113.5 g of nicarbazin/454 g of premix (2) and it is typically included in broiler starter feed at 113.5 g/ton of feed or at 90.8 g/ton in grower feed. In a previous report (3), the sensitivity of 26 field isolates of mixed coccidial species to nicarbazin by anticoccidial sensitivity tests (ASTs) showed that the great majority of coccidia field isolates remain sensitive to nicarbazin. During the past decade there has been an increased usage of nicarbazin but also of another anticoccidial that contains a lower level of nicarbazin (36 g/454 g of premix) plus 36 g of narasin/454 g of premix and it is sold under the trade name Maxiban® (2). This has raised the question as to whether the sensitivity of field isolates of coccidia to nicarbazin has remained unchanged in spite of the increased usage of nicarbazin resulting from increased use of both, nicarb and maxiban in broiler production. Since the last report on sensitivity of coccidial isolates to nicarbazin in 2008 (3), an additional 42 isolates of coccidia from a broad range of broiler operations across the United States have been collected and tested for sensitivity to nicarbazin by ASTs. The procedure for conducting ASTs has been described before (4). Briefly, in an AST, coccidia-free broiler chickens raised in wire floor cages and fed diets containing the corresponding anticoccidial are orally challenged with a measured dose of sporulated coccidial oocysts at 14 d of age. Six d post- challenge the birds and feed are weighed in order to calculate average weight gain, feed consumption and feed conversion and the birds are euthanized and its intestines removed and individually scored for gross coccidial lesions by the method of Johnson and Reid (5). The results are compared to the results obtained in replicates of broiler chickens that were challenged but were not fed any anticoccidial drug. The main parameters analyzed include: weight gain, feed conversion, mortality and intestinal lesion scores. As mentioned before, since the previous report (3) which detailed sensitivity results to nicarbazin from 26 field isolates of mixed coccidial species from broiler-producing areas across the United States, an additional 42 field isolates have been evaluated by the same procedure and have been added to the original 26 and will be presented in the results section of this manuscript. Only the sensitivity results to nicarbazin were analyzed in order to determine if during this 10 year period the sensitivity of *Eimeria* isolates from the field has changed.

## MATERIALS AND METHODS

**Sensitivity tests: Collection of field samples**. In addition to the 26 previous isolates, litter samples were collected from 42 broiler complexes from 2007 to 2013 representing a cross section of commercial broiler-producing companies in the United States. Samples were obtained from houses from the upper one-third of the litter representing the brood end of each house from flocks between 21-28 d of age. A one pound composite sample from each house was placed in a plastic bag, put on ice and submitted overnight to Southern Poultry Research, Inc .

**Preparation of litter samples**. Litter samples were mixed with feed at the rate of 100 g of litter per 100 g of feed and fed to unmedicated coccidia-free broiler chicks two to three wk of age. Droppings were collected six to eight d post-infection, homogenized and washed through a double layer of cheese cloth. The solids in the filtrate were separated by centrifugation for two minutes at 800 x g. The supernatant was discarded and the oocysts were re-suspended from the sediment in 2.5% w/v potassium dichromate and sporulated over a 72 h period at 30°C with forced aeration.

**Description of *in vivo* study**. The study consisted of a series 42 battery tests and was conducted from January 2007 to December 2013. Male Cobb x Cobb chicks were raised in coccidia-free Petersime battery units to 12 d of age. Assignment of treatments to cages was made by use of a random numbers table. Cages were blocked by location in the battery with block size equal to treatments by isolate (three cages per block). There

were three cages per treatment and eight chicks per cage for a total of 24 chicks per treatment. Treatments consisted of chicks fed diets containing either Nicarb (113 g/909 kg) or Maxiban (72 g/909 kg), or a non-medicated control. Batteries were in an environmentally-controlled structure with even illumination and a stocking density of 0.63 $ft.^2$/ bird.

**Procedure for *in vivo* study**. On d 12, day of test zero (DOT 0), chicks were weighed by cage. On d 14 (DOT 2) all chicks were infected orally by pipette with one mL of isolate suspension containing 100,000 sporulated oocysts. On d 20 (DOT 8) the trial was ended and birds were weighed by cage and total feed consumption by cage was determined. Birds were euthanized and lesion scored by cage according to the method of Jonnson and Reid (5). Lesion scores were determined for upper, middle, and cecal regions of the intestine and scores reported as average of all regions.

## RESULTS

A summary of the average results of the sensitivity tests for the combined 68 field isolates is presented in Table 1. There were no statistically significant differences ($P>0.05$) for mortality and therefore the data are not shown.

There were highly significantly ($P<0.0001$) differences between treatments in terms of weight gain, the challenged birds that were fed the non-medicated diet gained significantly less weight ($P<0.0001$) than those fed the diet supplemented with nicarbazin (257 vs. 336 g, respectively, Table 1). These results were consistent from year to year regardless of the year, for example, 2004 vs. 2013 (data not shown).

There were also highly significantly ($P<0.0001$) differences between treatments regarding feed conversion ratio, the challenged birds that were fed the non-medicated diet had significantly higher ($P<0.0001$) feed conversion ratio than those fed the diet supplemented with nicarbazin (2.006 vs. 1.584, respectively, Table 1 ). These results were consistent from year to year regardless of the year, for example, 2004 vs. 2013 (data not shown).

Finally, there were also highly significantly ($P<0.001$) differences between treatments regarding intestinal lesion scores, the challenged birds that were fed the non-medicated diet had significantly higher ($P<0.0001$) intestinal lesion scores than those birds fed the diet supplemented with nicarbazin. These results were consistent from year to year regardless of the year. Table 2 shows the average percent reduction in lesion scores per year and the total number of isolates per year.

## CONCLUSIONS

Isolations of coccidia from litter samples received from 68 broiler production complexes across the U.S. tested from 2004 through 2013 showed based on gross lesions that 79.6% contained *E. acervulina*, 19.2% *E. maxima* and 30.1% *E. tenella*.

Based on comparisons of sensitivity testing from 2004 to 2013 and the widely accepted interpretation of sensitivity testing results that states that reductions in gross lesion scores of 50% or greater mean that the coccidial isolates are sensitive, between 30-49% mean that the coccidial isolates are intermediately sensitive and less than 30% mean that the coccidial isolates are resistant. Even though there may be numerical fluctuations from year to year, it is evident that, regardless of the year, nearly all isolates of *Eimeria* from the field still remain fully sensitive to nicarbazin.

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**Table 1**. Average body weight, feed conversion ratio and lesions scores at 20 d (DOT8) of unchallenged controls and broiler chickens challenged with composite field isolates of *Eimeria* from 68 anticoccidial sensitivity tests (ASTs) conducted from 2004 to 2013.

| Parameter(s) | Non-medicated unchallenged | Non-medicated challenged | Nicarbazin challenged |
|---|---|---|---|
| Body weight gain (g) | 368.3[a] | 257[c] | 336.3[b] |
| Feed conversion ratio | 1.506[c] | 2.006[a] | 1.584[b] |
| Lesion scores, upper | 0[c] | 2.547[a] | 0.732[b] |
| Lesion scores, mid | 0[c] | 2.410[a] | 0.567[b] |
| Lesions scores, ceca | 0[c] | 2.472[a] | 0.219[b] |

[a,b] Means in each row not sharing the same superscript are significantly (P<0.05) different.

**Table 2**. Average percent reduction in lesion scores by region of the intestine and by year due to nicarbazin*.

| | Intestinal Region | | |
|---|---|---|---|
| Year | Upper (N) | Middle (N) | Cecal (N) |
| 2004 | 67.5 (9) | 46.7 (2) | 97.6 (7) |
| 2005 | 67.4 (6) | - (0) | - (0) |
| 2006 | 71.4 (9) | 81.3 (2) | 100 (1) |
| 2008 | 89.4 (2) | - (0) | - (0) |
| 2009 | 73.8 (14) | 70.5 (2) | 90.4 (8) |
| 2010 | 73.8 (3) | - (0) | - (0) |
| 2011 | 100 (1) | 86.2 (1) | 89.1 (2) |
| 2012 | 88 (2) | - (0) | - (0) |
| 2013 | 68.3 (9) | 75.9 (3) | 74.4 (4) |

* When compared to non-medicated challenged controls.
Interpretation: 50% or greater = sensitive; 30 to 50% intermediate; <30% = resistant

# WHITE STRIPING APPEARANCE ALONG GROWTH ON BROILER CHICKENS TREATED WITH DIFFERENT COCCIDIOSIS CONTROL PROGRAMS

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## INTRODUCTION

White striping (WS) is an alteration of broiler muscle characterised by the presence of white striations parallel to the direction of muscle fibers. This condition is becoming increasingly important in broiler production because it affects the acceptance of raw meat by the consumer and its physicochemical characteristics, thus decreasing the economic value (1). In this study the age of WS onset in broiler chickens and the effect of diet and of different coccidiosis control programs on WS prevalence at slaughter, were evaluated.

Four groups of 180 chickens were randomly allotted to four experimental treatments: control (C), coccidiostat (Cox), vaccinated (V), and vaccinated fed with a low energy diet (VLE). The birds were weighed at 12, 25, 42, and 51 d of age. Twenty animals per treatment were sacrificed at 12 and 25 d of age, whereas the remaining chickens at 51 d. Breasts were categorized based on the degree of WS following the lesion score proposed by Kuttappan et al. (2): 0=absence (normal breast), 1=moderate (breast with white lines less than 1 mm thick), 2=severe (breast with white lines more than 1 mm thick). For histological evaluation, up to now 34/80 (d 12) and 46/80 (d 25) breast samples were examined.

At 12 d of age all breasts were classified as normal (WS score 0), whereas at 25 d of age WS was macroscopically evident with score 1 in a limited number of birds (3/20 group C, 2/20 group Cox, 3/20 group V, 1/20 group VLE).

Histologically, myopathic changes were found in 29/34 (85%) of samples at d 12, and 46/46 (100%) of samples at d 25. At d 12, breast muscles were affected by fiber size change, and multifocal mild degeneration/necrosis, while at d 25 these alterations were increasingly severe and associated with heterophilic and macrophagic infiltration, early interstitial fibrosis, and fat infiltration.

The absence of macroscopic lesions at d 12 associated with the presence of early and mild histological lesions in some samples indicates the onset of WS around 12 d.

At 51 d of age the prevalence of WS was above 90% in all groups, and the prevalence of WS score 2 was 77.6%, 64.4%, 62.6% and 61.6%, for Cox, VLE, V and C, respectively, being significant ($P<0.001$) the difference Cox *vs* V and C.

The coccidiostat effect on the increase in WS severity seems attributed to the higher initial birds' growth.

This study provided a new perspective for the control of WS prevalence and a starting point for further studies.

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

# PHENOTYPIC, GENOTYPIC AND PATHOGENICITY ASSESSMENT OF *SALMONELLA* INFANTIS STRAINS ISOLATED FROM POULTRY

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## SUMMARY

*Salmonella enterica* subspecies enterica serovar Infantis has been associated with human illness linked to contamination of poultry products. However, in the US *Salmonella* Infantis human outbreaks had been linked primarily to live poultry. The presence of multidrug resistant strains circulating in poultry as well as the emergence of this serotype possibly associated to interventions directed to other *Salmonella* serotypes around the world supports the relevance of *Salmonella* Infantis in public health. The present study evaluated the phenotypic, genotypic and pathogenic characteristics of *Salmonella* Infantis strains isolated from commercial poultry in the US. Phenotype microarray (PM), including antibiotic susceptibility profiles, genome analysis and virulence assessment in poultry were performed and results are presented and discussed. Even though some differences in virulence were observed, high challenge doses of *Salmonella* Infantis were required for colonization of chicken internal organs with no mortality or any other clinical sign associated with infection.

*Salmonella enterica* subsp. *enterica* serovar Infantis is classified as O:7 ($C_1$) serogroup under the White- Kauffmann-Le Minor scheme. This *Salmonella* serovar has been associated to salmonellosis in humans linked to food products (1,4). In the U.S. human infections were mainly linked to direct contact with live poultry (2). The emergence on multidrug-resistant (MDR) strains has been reported (3) therefore representing a continuous hazard to public health. This study investigated the phenotypic, genotypic and pathogenicity characteristics of *Salmonella* Infantis strains isolated from poultry environments in the US and assessed their antimicrobial susceptibility. Our goal was to contribute to the understanding of *Salmonella* Infantis specific characteristics that may be used to control its dissemination and infection in poultry thus reducing their impact on public health.

## MATERIALS AND METHODS

***Salmonella* Infantis strains.** A total of 10 *Salmonella* Infantis strains were used in this study. Eight strains isolated from U.S poultry environmental samples or submitted to Ceva Biomune Laboratory Services in 2014 were compared. Two *Salmonella* isolates from Europe were used as references. Strains are preserved in the Ceva Biomune *Salmonella* stock library.

**Microbial growth an initial characterization.** All the strains were cultured using standard microbiological media, assessed for motility, biofilm formation and general biochemical characteristics (API 20E). *Salmonella* serotype was confirmed by the Intergenic Sequence Ribotyping (ISR) method described by Guard et. al (5).

**Phenotype analysis.** Phenotyping was assessed using the PM Technology (Biolog) on an OmniLog instrument. A total of 2,870 substrate utilization assays for carbon, nitrogen, phosphorus, sulfur, nutrient, and peptide nitrogen sources along with osmolytes, pH and antimicrobial susceptibility tests were utilized. The Biolog PM data analysis was carried out using an OmniLog phenotype microarray (OPM) software package. Respiration curve height values relative unit (RU) were used for comparisons between strains.

**Whole Genome Sequencing.** Whole Genome Sequencing (WGS) was conducted using the OneTouch v2 and Ion Torrent PGM (Life Technologies, Carlsbad, CA). Briefly, the *Salmonella* Infantis genomes were sheared, barcoded, amplified and sequenced using the Ion Torrent 400bp sequencing workflow according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). Sequence assembly and analysis was performed using the bioinformatics tools from DNASTAR (Madison, WI).

**Pathogenicity in chickens.** Five *Salmonella* Infantis strains were inoculated in one-day-old SPF Leghorn chickens. Animal experiments observed the appropriate care and use of experimental animal's

guidelines from the Office of Laboratory Animal Welfare (OLAW). Signs of disease and mortality were recorded up to 14 d post infection. Two wk post-challenge the birds were euthanized and *Salmonella* re-isolated from liver and spleens.

**Data analysis.** Variability in phenotypes was assessed on PM data (RU) with standard deviations (SD) between strains greater than 34. Antimicrobial sensitivity was indicated when less than 100 RU were obtained and resistance for values equal or greater than 100 RU. Genome sequences were compared based on the detection of 100% non-synonymous single nucleotide polymorphisms (SNP) against the *Salmonella* enterica subsp. enterica serovar Enteritidis str. P125109 genome (NCBI Reference Sequence: NC_011294.1). Gene and PM reaction association, when established, were conducted searching KEGG Mapper (www.genome.jp/kegg/) and peer-reviewed publications.

## RESULTS

The standard microbiological evaluation did not show major differences between strains. They were classified as generally motile with weak and slow biofilm formation, and inositol negative *Salmonella* spp. Two different genotypes were identified using the ISR method (Table 1).

**Phenotype analysis.** Metabolite utilization showed high similarity between the U.S *Salmonella* Infantis strains. All the strains evaluated showed tolerance to up to 6% NaCl, 5% sodium lactate, 15% ethylene glycol, 200mM sodium phosphate (pH 7), and to a pH range between 5 and 10. Only one U.S. strain (LS14-1611) differed in few carbon (e.g. D-Xylose, Inositol, Gluconic acid), nitrogen (Tyramine) and nutrient supplement (e.g.D-Biotin, carnitine, choline) utilization therefore behaving more similar to EU strain LS14-1647-4. Of all the strains analyzed, EU strain LS14-1647-4 utilized with the least efficiency several carbon, nitrogen, phosphorus, sulfur and nutrient supplements. U.S *Salmonella* strains showed significant resistance to amoxicillin, ceftriazone, gentamicin and erythromycin while the EU strains showed extended resistance to tetracyclines, ciprofloxacin, nalidixic acid and sulfathiazole.

**Whole genome sequencing.** Compared to the *Salmonella* enterica subsp. enterica serovar Enteritidis str. P125109 genome, all *Salmonella* Infantis strains evaluated (U.S. and E.U.) showed high similarity evidenced by the presence of a large group of genes (core) with the same SNP content (Figure 1). *Salmonella* Infantis strains LS14-1611 (US) and LS-1647-4 (EU) resulted in the largest numbers of non-synonymous SNPs (226 and 64, respectively) located in open reading frames (ORF). Altered genes associated to metabolic pathways were between 38-47%. Genes associated to virulence were between 8-25%. Antimicrobial, stress response and sensor associated genes represented less than 5%. Fimbria, flagella and *Salmonella* Pathogenicty Island (SPI) genes represented 57% of the virulence associated genes.

**Pathogenicity in chickens.** As shown in Table 1, all *Salmonella* Infantis strains yielded low or no mortality. *Salmonella* Infantis was generally re-isolated from more than 85% of the cases except for strain LS14-1647-4 (EU) which showed low recovery from liver and spleens (15%) two wk post-infection.

## DISCUSSION

*Salmonella* Infantis isolated from poultry environments in the U.S. were compared by phenotyping, genotyping and pathogenicity in chickens. The results of this study support high clonality of *Salmonella* Infantis isolates in the U.S. However, we were able to differentiate at least two types of strains that differed in their metabolic characteristics and ORF alterations. *Salmonella* Infantis LS14-1611 US strain showed some phenotypic similarities to EU strain LS14-1647-4. The latter showed greater metabolic limitations which correlated with a large number of altered ORFs associated to metabolic pathways and reduced pathogenicity. Moreover, the differences in ISR genotypes did not correlate to pathogenicity. Even though, gene disruptions do not necessarily imply alteration in protein functionality, trends observed from the analysis suggested adaptation to stress conditions and invasion through changes in other than the classical virulence associated genes. The antibiotic susceptibility was not clearly supported by the whole genome sequencing results possibly explained by the fact that only genomic DNA was tested. Unconfirmed associations between disruption in ORFs and phenotyping results may explain deficiencies in cobalamine (pduF, cobU, cysG), propionic acid (gltA), tyramine (potE, cadA), D-biotin (accC), L-valine (ilvC) utilization or tolerance to NaCl (yjbJ) and pH (rstA, cadA). Further studies are required to validate gene to phenotype correlations.

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**Figure 1.** Non synonymous Single Nucleotide Polymorphisms (SNPs) shared between strains.

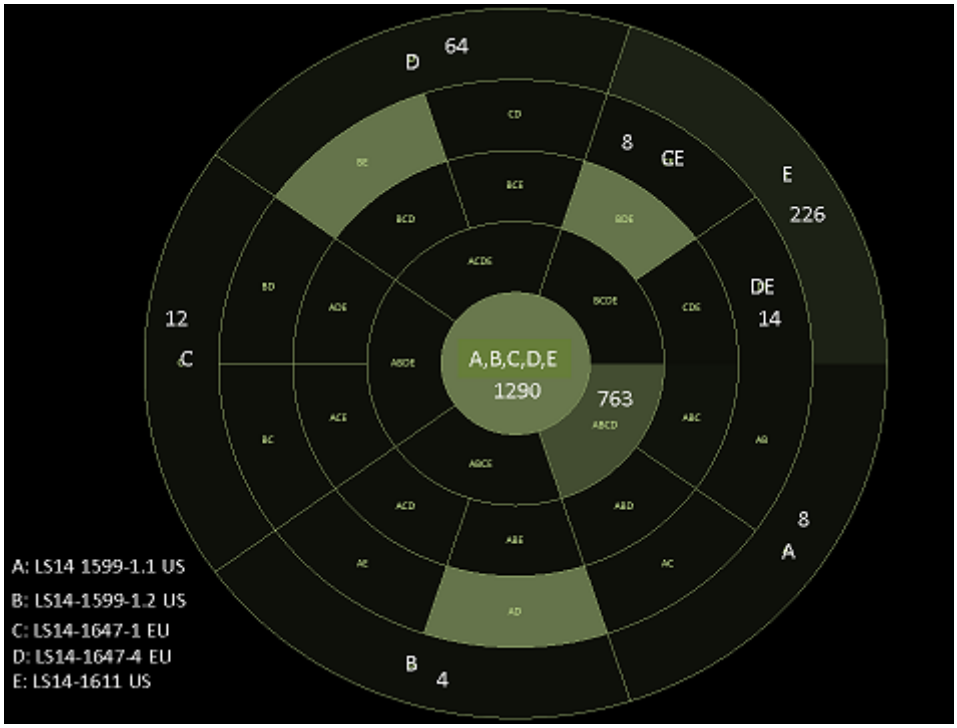

**Table1.** *Salmonella* Infantis re-isolation from liver/spleen samples two weeks post-experimental challenge in 1 day-old SPF Leghorn chickens.

| Strain ID | Origin | ISR genotype | N° Birds | Challenge Oral Dose CFU/0.25 mL | % Mortality | N° Positives/Total (%) |
|---|---|---|---|---|---|---|
| LS14-1599-1.1 | US | C&T_500bp_Infantis | 25 | 8.20E+07 | 8% | 21/23 (91%) |
| LS14-1599-1.2 | US | C&T_500bp_Infantis | 30 | 6.00E+07 | 10% | 23/27 (85%) |
| LS14-1647-1 | EU | REF_Infantis_500bp_9381 | 20 | 5.90E+07 | 0% | 18/20 (90%) |
| LS14-1647-4 | EU | REF_Infantis_500bp_9381 | 20 | 7.80E+07 | 0% | 3/20 (15%) |
| LS14-1611 | US | REF_Infantis_500bp_9381 | 25 | 8.50E+07 | 0% | 23/25 (92%) |

*US: United States of America; EU: European Union, CFU: Colony Forming Units, ISR: Intergenic Sequence Ribotyping.

# SAFETY AND EFFICACY OF A CHICKEN EMBRYO ORIGIN LIVE FOWL POX VIRUS VACCINE IN ONE DAY OLD COMMERCIAL TURKEYS

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## SUMMARY

A freeze dried chicken embryo origin live fowl pox virus vaccine was tested for its safety and efficacy in one d old turkeys by administering the vaccine subcutaneously. Following vaccination, the turkeys were observed up to three wk for vaccine related mortality and adverse reactions. Efficacy was evaluated by challenging the turkeys against a virulent challenge strain of fowl pox. The vaccine proved to be safe for subcutaneous administration in one d old turkeys. None of the vaccinated turkeys revealed any adverse reactions or mortality associated with the vaccine. The vaccine also protected more than 98% of the turkeys against fowl pox virus challenge.

## INTRODUCTION

Poxvirus infection in commercial turkeys is recognized as one of the economically important diseases in turkey industry. The disease is characterized by transient epithelial hyperplasia, inflammation and necrosis. The cutaneous form of the disease is characterized by appearance of nodular lesions on the comb, wattle, eyelids and other nonfeathered areas of the body. The diphtheritic form causes typical cankers or diphtheritic lesions on the mucous membrane of the mouth, tongue, upper part of trachea, cloaca and vent (2, 3, 4). Typically, turkeys are vaccinated with fowl pox vaccine at four to 10 wk of age. Since fowl pox vaccine does not produce lasting immunity in turkeys, revaccination is often done when needed (1). To the authors' knowledge, no published information was yet available on the safety and efficacy of the commercially available fowl pox vaccine in turkeys as young as one d old. The objective of this study was to evaluate the safety and efficacy of a commercially available fowl pox vaccine manufactured by Hygieia Biological Laboratories in one d old commercial turkeys.

## MATERIALS AND METHODS

One group of 55 turkeys (Breed: Nicholas) were vaccinated with Hygieia's fowl pox vaccine, live virus (Serial # FPM001) and another group of 50 turkeys were kept as unvaccinated controls. Prior to use, the lyophilized vaccine was rehydrated with sterile diluent to deliver one dose in 0.2 mL. One vial of vaccine containing 1000 doses was rehydrated and diluted with 200 mL diluent. Each turkey was vaccinated with 0.2 mL of vaccine subcutaneously in the back of the neck. Following vaccination, the vaccinated and control turkeys were housed in separate cages and provided food and water *ad libidum* during entire period of observation.

All turkeys (vaccinates and controls) were observed daily up to three wk post vaccination for vaccine associated mortality, adverse reactions and development of clinical signs of virulent pox virus infection. At three wk post vaccination, all turkeys (vaccinated and controls) were challenged against virulent fowl pox virus (USDA strain) to evaluate vaccine induced immunity. The challenge virus was administered through wing web using a double needle applicator. At six d post challenge, each turkey was checked for development of pox lesion (take) at the site of challenge virus administration. Absence of a palpable "take" indicated protection against pox challenge.

## RESULTS AND DISCUSSION

The vaccine proved to be safe for subcutaneous administration in one d old turkeys. None of the vaccinated turkeys revealed any adverse reactions or mortality associated with the vaccine as observed up to three wk post vaccination. One out of 55 vaccinated turkeys died at four d post vaccination due to a cage accident. The remaining 54 turkeys remained well and healthy until the termination of the experiment. No other abnormality was observed in the vaccinated birds in respect to their growth and health status. In the control group, six out of 50 turkeys died within the first five d of vaccination due to a cage accident. The remaining 44 turkeys

remained well and healthy until the termination of the experiment.

The vaccine induced excellent protection in the vaccinated turkeys. 53 of 54 vaccinated turkeys (>98%) resisted challenge against the USDA challenge virus. Whereas, 0 of 44 unvaccinated control turkeys (0%) protected against challenge virus infection (Fig. 1). Following challenge, all unvaccinated control turkeys became positive for pox lesions.

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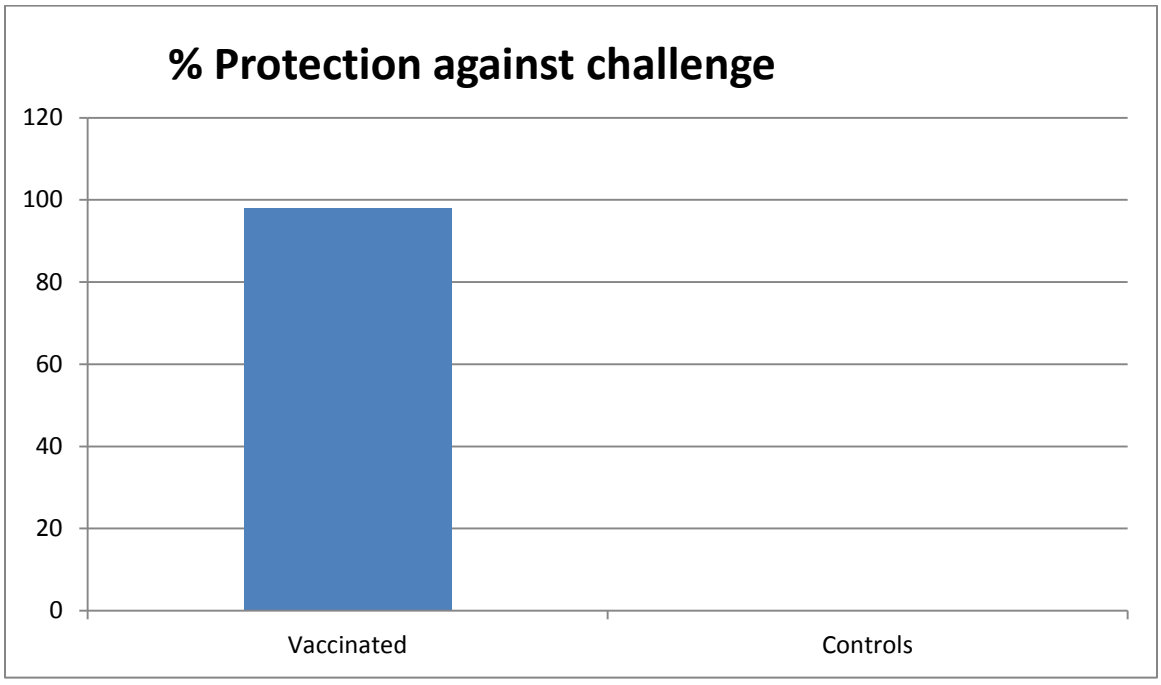
**Figure. 1**. Protection against fowl pox virus challenge.



# CASE REPORT: OUTBREAK OF TYPE C BOTULISM IN COMMERCIAL LAYER CHICKENS

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## SUMMARY

This report describes an outbreak of type C botulism in two organic, free-range commercial layer flocks in the Midwest. Hens affected were 64-wk-old Hy-Line Brown hens and 34-wk-old Hy-line Brown hens with approximately 20,000 birds per house. Mortality over the two wk of investigation was estimated to be up to 8% and 2.8%, respectively, with birds acting listless, lethargic, and depressed. Clinical signs consisted of progressive paralysis, and severely affected birds were moribund and laterally recumbent. Hens had ruffled feathers that easily epilated, with loss of muscular tone in the neck, tail, and wings. Hens had closed eyes and were reluctant to move. There were no significant gross or histopathologic lesions. Intestinal samples were submitted to the Pennsylvania Botulism diagnostic laboratory for Real-time polymerase chain reaction (Rt-PCR), and were positive for type C *Clostridium botulinum* toxin. Speculations on the source of the botulinum toxins include poor mortality removal leading to cannibalism of decomposing carcasses, as well as birds on the farm having access to putrid carcasses in the compost pile from a hole in their outdoor access fence.

## INTRODUCTION

Botulism may be caused by toxicoinfection or by ingestion of pre-formed toxins of *Clostridium botulinum* in feed, carcasses, or maggots harboring high concentration of botulinum toxin. Type C toxin is associated with most outbreaks in poultry, although other types have been documented in literature (2). Clinical signs may appear within a few hours to a few d post-exposure, and may include drowsiness, weakness, and paresis, resulting in progressive flaccid paralysis of the legs, wings, neck (limberneck), and eyelids. In many chickens, the feathers tend to be loose and easily epilate from the neck. Most visibly affected birds die either from cardiac and/or respiratory failure. Suspicion of botulism is based on history, clinical signs, and absence of gross lesions upon necropsy examination. Botulism is confirmed by detection of the botulinum toxin using the mouse bioassay with serum neutralization or enzyme-linked immunosorbent assay (ELISA), or PCR to detect the neurotoxin gene. Numerous reports have described outbreaks in commercial broiler (1, 3), turkey (7), and layer (2, 8) operations housed indoors, and are mostly due to single-farm-related problems based on rearing environments (2). This report describes two outbreaks of type C botulism in a free-range, commercial organic layer operation in the Midwest.

## CASE HISTORY

The first outbreak occured in June 2014 on a free-range, organic, commercial layer farm. These hens were 64-wk-old Hy-Line Brown hens of 19,372 birds. A second, free-range, organic flock of 34-wk-old Hy-line Brown hens experienced similar problems to a lesser degree. Three-day-mortality of the first flock prior to submission to the Purdue Animal Disease Diagnostic Laboratory (ADDL) was 41, 93, and 10. Birds posted in the field had mucus-filled intestines with enteric nematodiasis and white diarrhea, and all birds were in production with thick abdominal fat pads. Spleens were slightly smaller, and hearts appeared grossly enlarged.

## CLINICAL SIGNS

Clinical signs in the affected hens were those of progressive paralysis, with severely affected birds being moribund and were laterally or sternally recumbent. The hens were paretic, with soiled vents due to the diarrhea. The hens had marked loss of tone in the tails and wings, as well as ventroflexion and flaccid paralysis of the neck (limberneck). Their eyes were shut, and the hens were reluctant to move.

## GROSS AND MICROSCOPIC FINDINGS

Ten 64-wk-old Hy-line Brown hens were presented to the Purdue ADDL for necropsy. All ten birds were moribund and were listless, depressed, lethargic, and paretic, and were reluctant to move. The hens had ruffled feathers that easily pulled out, with loss of tone in the tails and wings, and closed

eyes. All ten birds were in good body condition with adequate subcutaneous fat and pectoral muscling, and all birds were in production. There were no significant findings upon gross examination, with the exception of enteritc parasitism, with nematodes compatible with *Capilliaria* spp. and *Ascaridia* spp. Three out of ten birds had right cystic oviducts, and one bird had a leiomyoma along the mesentery of the mesosalpinx. Five out of ten birds had multiple 1 to 2 mm pale white to tan foci on the liver that were histologically unremarkable.

Histopathologic examination revealed minor incidental findings that may have contributed to the morbidity of the flock but were not consistent with clinical findings observed antemortem.

Choanal swabs from the birds were negative for avian influenza using an avian influenza type A direct antigen detection test kit (*Flu* Detect™, Synbiotics). There were no significant changes in the ELISA serum titers for Newcastle disease virus since vaccination. Brain samples were sent to test for sodium levels by atomic absorption spectrophotometry, and the result was 1750.82 ppm (normal levels – 1600 - 1710 ppm, toxic levels in poultry brain = 1900 - 2140 ppm).

**RT-PCR.** Pooled samples from the affected flock including intestines, intestinal contents, crop and gizzard contents, were sent to the Pennsylvania botulism diagnostic laboratory to detect the neurotoxin gene of *C. botulinum*.

Positive controls for *C. botulinum* types A and B (bei Resources) were acquired from commercially available genomic DNA to develop a duplex qPCR assay for botulinum neurotoxin types A and B. A singleplex qPCR assay was used for botulinum neurotoxin type C using positive field sample DNA.

Samples tested positive for type C botulism by Rt-PCR and negative for types A and B.

## FIELD OBSERVATIONS

A field visit to the affected now 65-wk-old brown flock was performed by the avian veterinary service at the Purdue ADDL. The flock was still experiencing clinical signs typical of botulism, with sporadic deaths (<3%/week). Hens were housed in a cage-free facility with outdoor access to a fenced-in pasture. Morbidity was estimated to be approximately 10%/week. Sporadic birds were experiencing similar clinical signs as the birds observed upon necropsy, either found laterally recumbent or squatting onto the litter, with feathers that easily epilated upon handling. Several putrifying chicken carcasses were found within the house, along with numerous floor eggs and many hens exhibiting egg-eating behavior.

A second affected Hy-Line Brown flock (34-wk-olds) at 35 wk of age had similar clinical presentations with acute death loss reaching 1.8%/wk with high cull rates. Morbidity was estimated to be >5%/wk. The houses were constructed similarly, with outdoor access to a fenced-in pasture. Both houses had mismanagement concerns, with inadequate removal of daily mortality, as well as high numbers of floor eggs. In the second house, there was a hole in the outdoor access fence leading to the mortality compost pile, with evidence of birds cannibalizing autolyzed bird carcasses and the hens dragging them back into the pasture. In addition, the compost pile lay flat along the concrete just adjacent to the pasture, and the outdoor pasture was flooded.

## DISCUSSION

Total cumulative mortality, including culled birds, reached 23.6% (4,747 hens) at 86 wk in the first flock (64-wk-olds), and 12.12% (2,605 hens) in the second flock (34-wk-olds) at 60 wk (Hy-Line brown performance standards = 6.1% cumulative mortality at 80 wk, 3.5% at 60 wk)(4). Management interventions applied on these farms to prevent further death loss included better removal of mortality and administration of oregano oil through the water. The use of oregano oil and other organic acids are thought to improve intestinal health by reduction of pH and bacterial growth intolerant to pH changes. However, prophylactic management practices such as carcass removal and improving biosecurity (i.e. fixing breach in the outdoor access fence) are far superior to treatment. Although the two cases were isolated incidences, similar management concerns were speculated to be the source of botulism in the two flocks.

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# REDUCING THE INCIDENCE OF BACTERIAL CHONDRONECROSIS WITH OSTEOMYELITIS IN BROILERS WITH A DIETARY COMBINATION OF *BACILLUS SUBTILIS* C-3102 (CALSPORIN®) DIRECT FED MICROBIAL AND IMW50™ YEAST CELL WALL PREPARATION

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## INTRODUCTION

Bacterial chondronecrosis with osteomyelitis (BCO) is a leading cause of lameness in commercial broilers produced in North America (1, 2, 9). The lesions most often occur in the proximal femora and tibiae and are associated with osteochondrotic microfractures and clefts in rapidly growing broilers. Bacteria distributed hematogenously exit the bloodstream and colonize the structurally compromised regions of the bone. As the bacteria proliferate and an immunologic response occurs, the result is necrosis and caseous exudate (4, 8, 9). Multiple bacteria of various species have been isolated from BCO lesions. Most predominantly, *Staphylococcus* spp., *Escherichia coli*, and *Enterococcus* spp. have been identified. (4, 9).

The portal of entry by pathogenic bacteria into the systemic circulation is considered to be through compromised tight junctions of the intestine. Stress and the composition of the intestinal microbiome appear to influence the integrity of the intestinal mucosa (6, 7) of bacterial translocation across the intestinal mucosa has been experimentally reproduced at a high incidence in male broilers reared on wire flooring. The wire flooring enhances the torque and shear stress on the growth plates of the proximal femora and tibiae. A diverse population of bacteria has been identified in association with this syndrome.

The hypothesis is that bacteria enter the blood stream by translocating from the intestinal tract. The hematogenous circulation of these bacteria infect micro-fractures and clefts in the growth plate region of rapidly growing bones. In this study, BacPack™ 2X was fed prophylactically, starting at one day-of-age to reduce intestinal bacterial translocation. This treatment was compared to hatch-mates that received a control diet and were reared in the same environmental and management conditions.

BacPack 2X is a proprietary blend of Calsporin® (*Bacillus subtilis* C-3102) and yeast cell wall product IMW50™, which is incorporated into rations prior to feed pelleting. BacPack 2X is resistant to pelleting temperatures up to 90°C. Male broilers reared on wire flooring resulted in a significant ($P \leq 0.05$) increase in the incidence of BCO compared to hatch-mates reared on wood shavings. The prophylactic feeding of BacPack 2X resulted in a significant ($P \leq 0.05$) reduction in the incidence of BCO in broilers reared on wire flooring at 56 d of age compared to broilers receiving a control feed. The feeding of BacPack 2X also significantly ($P \leq 0.05$) delayed the age of onset of BCO compared with broilers fed the control feed. Clinically healthy broilers that had been reared on wire flooring for 56 d had higher body weights when they had been fed the diet containing BacPack 2X. The results of this study indicate that the prophylactic feeding of BacPack 2X provides a mechanism toward the reduction of BCO-related lameness in broilers.

## MATERIALS AND METHODS

Environmental chambers were constructed and managed as previously reported (9). Feed (crumbles through wk three and pellets thereafter) and water were provided *ad libitum*. Corn and soybean meal-based broiler starter feed formulated to meet minimum National Research Council (1994) standards for all ingredients was provided as the control feed in chambers 1, 3, 5, and 7. The feed containing BacPack 2X was provided in chambers 2, 4, 6, and 8, and consisted of control feed mixed prior to pelleting with 1 lb/ton of Quality Technology International Incorporated's BacPack 2X, which contains the MOS beta-glucan yeast cell wall

prebiotic IMW50 plus the Calsporin *Bacillus subtilis* C-3102 probiotic. Feed specifications were determined by QTI's technical staff, and the feed was manufactured by the University of Arkansas Poultry Research Feed Mill.

Cobb 500 male chicks were obtained unvaccinated from a commercial hatchery and initially were placed at 61/pen. They were culled to 50/pen on d 14, yielding a density of 1 $ft^2$/chick. Beginning on d 15, all birds were observed daily to detect the onset of lameness. Affected broilers had difficulty standing, exhibited an obvious limping gait while dipping one or both wing tips and, if not removed, became completely immobilized within 48 h. Birds were humanely euthanized as soon as the onset of lameness was noticed, and were necropsied within 20 min post-mortem. Birds found dead were necropsied to ascertain the cause of death and assess leg lesions. All birds that died or developed clinical lameness were recorded by date and pen number, and then they were necropsied and assigned to one of the following categories):

**Normal F** (no macroscopic abnormalities of the proximal femur)

**FHS** (proximal femoral head separation or epiphyseolysis)

**FHT** (proximal femoral head transitional degeneration)

**FHN** (proximal femoral head necrosis)

**Normal T** (no macroscopic abnormalities of the proximal tibia)

**THN** (mild proximal tibial head necrosis, a sub-category of BCO in the tibiotarsus)

**THNs** ("severe" THN in which the growth plate was imminently threatened or damaged)

**THNc** ("caseous" THN in which caseous exudates or bacterial sequestrae were macroscopically evident)

**TD** (tibial dyschondroplasia)

**Lame-UNK** (lameness for unknown or undetermined reasons).

At the end of the experiment on d 56, representative survivors that appeared to be clinically healthy were euthanized, weighed and necropsied to assess sub-clinical lesion incidences (n = 20 each for the litter flooring chambers; 10 each for the wire flooring chambers).

## STATISTICAL ANALYSIS

The statistical application was performed as previously reported (9).

## RESULTS

Between d 15 and 56, lameness due to KB, TW, TD, and LAME-UNK were minimal, and these non-BCO causes of lameness did not differ between diet treatments. Lameness overwhelmingly was attributable to BCO lesions of the proximal femora and tibiae. Incidences of BCO lameness and total lameness were significantly lower (P = 0.003) in broilers fed the BacPack 2X than in those fed the Control diet.

Regarding the time course of lameness progression, broilers fed the Control diet had higher incidences of BCO on d 36 (P = 0.036) through 43, and on d 46 through 56 (P = 0.001) when compared contemporaneously with broilers fed the BacPack 2X diet. The diet treatment groups did not differ (ns, P = 0.090) on d 44 and 45. The onset of BCO occurred earlier in broilers fed the Control diet, and the overwhelming majority of BCO lameness developing during the $7^{th}$ and $8^{th}$ wk in both of the diet treatment groups. Variation in total BCO incidences among the individual environmental chambers were observed. The lowest incidence among the wire flooring chambers receiving the control diet (36%, CW chamber 3) numerically exceeded the highest incidence for the wire flooring chambers receiving the BacPack 2X diet (32%, BW chamber 4). We consider this range of variability to be typical based on five years of prior experience with the wire flooring model.

Within each flooring category, broilers fed the Control and BacPack diets differed minimally, with the exception that survivors on litter flooring had higher incidences of normal proximal femora and lower incidences of FHT when they had been reared on the Control diet. Independent of diet treatment, broilers that survived eight weeks on wire flooring had significantly higher incidences of proximal tibial BCO lesions (THN+THNs+THNc) than the survivors reared on litter. Regarding, body weights of the clinically healthy survivors, broilers reared on litter and fed the Control diet were the heaviest. Broilers reared on wire and fed the Control diet were the lightest. Clinically healthy survivors that had been reared on wire flooring for 56 d had higher body weights when they had been fed the BacPack 2X diet instead of the control diet ($4.03 \pm 0.05$ vs. $3.81 \pm 0.07$, respectively; P = 0.012). Pooling these data by diet treatment regardless of floor type eliminated all differences in eight wk body weights ($3.99 \pm 0.06$ for Control vs. $4.02 \pm 0.04$ for BacPack 2X; P = 0.645).

## DISCUSSION

The wire flooring model successfully triggered significant incidences of BCO lameness in Cobb 500 male broilers. In the present and previous studies most of the lameness triggered by wire flooring developed between six and eight wk of age, as has been reported for field outbreaks of BCO (2, 3, 9). In this regard it is noteworthy that when chick quality is an issue, then osteomyelitis and typical BCO lesions can occur during the first few days post-hatch. Our standard protocol incorporates heavy culling on d 14, which is intended to minimize the appearance of clinical BCO prior to d 40. Accordingly, it is important to note that BCO lameness began to accumulate during the 4$^{th}$ and 5$^{th}$ wk for broilers fed the Control diet, but not until the 6$^{th}$ wk for broilers fed the BacPack diet.

BacPack 2X in the diet clearly delayed the onset of BCO lameness, and BacPack 2X also significantly reduced the eight wk incidence of BCO when compared with the Control diet. Virtually all of the BCO lameness occurred in broilers reared on wire flooring, and within the wire flooring category the eight wk survivors that had been fed BacPack 2X were significantly heavier than the survivors that had been fed the Control diet. The efficacy of BacPack 2X clearly cannot be attributed to restricting the growth of broilers reared on wire flooring. Also for broilers reared on wire flooring the BCO lesion incidences did not differ between diet treatments, either for the birds that developed BCO lameness or for the survivors at eight wk of age. Accordingly, although the specific biological mechanism remains to be determined, adding BacPack 2X to the diet did enable significantly more birds to survive eight wk on wire flooring, and those BacPack 2X survivors were heavier than survivors fed the Control diet.

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

# GENOTYPIC ANALYSIS OF AVIAN REOVIRUSES FROM CLINICAL SUBMISSIONS OF VIRAL ARTHRITIS

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**SUMMARY**

Starting in the fall of 2011, increased cases of tenosynovitis associated with variant avian reoviruses have been reported in the United States and other countries. Genotypic analyses of reovirus field isolates were performed and these isolates grouped in one of the five previously described genotypic clusters based on the amino acid sequence of the Sigma C. Two predominant variant groups were identified and generated distinct subgroups within two separate clusters. The predominant groups were identified as Group 1 (Cluster 5) and Group 2 (Cluster 1) VA (viral arthritis) variants. Briefly, the amino acid sequences of the reovirus sigma C for US commercial vaccine strains are >99% similar to each other. Within cluster 5, Group 1 VA variants are >97% similar to each other and 80% similar to Australian reovirus isolates RAM-1 and Somerville. Within Cluster 1, Group 2 variants are 75-80% similar to other reoviruses in this cluster. In addition, the amino acid sequence of Group 1 variants are less than 50% similar US commercial vaccines strains S1133, 2408, 1733 and 2177, while Group 2 variants are 80% similar to vaccine strains. The range of similarity in isolates belonging to Cluster 2 is 69-98%, 84-95% for isolates in Cluster 3 and 58-92% for isolates within Cluster 4.

During fall 2014, a surge in clinical cases of reovirus-induced tenosynovitis was observed, even in progeny from breeders receiving mono- or multivalent reovirus autogenous vaccines. Genotypic analysis of the new field isolates suggest additional variant groups (Cluster 4 and Cluster 3) have emerged that are genetically distinct from Group 1 and 2 VA variants and commercial reovirus vaccines. Relative distribution of variant reovirus field isolates within genetic Clusters 1-5, from clinical cases of tenosynovitis, was evaluated from 2012-2014. Group 1 VA variants (Cluster 5) have remained the predominant variant during this time, however with the increased usage of Group 1 isolates in autogenous vaccines, number of isolations are lower than in the two previous years. Group 2 VA variants (Cluster 1), while second most predominant isolate in 2012, decreased in number of isolations in 2013 and increased in isolations along with field isolates belonging to Clusters 2 and 4 in 2014.

# ORNITHOBACTERIOSIS CAUSING HIGH MORTALITY AND NEUROLOGICAL SIGNS IN CALIFORNIA BROILER CHICKENS

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## SUMMARY

Sudden and very high mortality associated with neurological signs were the reasons for multiple submissions of 35-45 d old, meat type chickens to the California Animal Heath & Food Safety Laboratory System, Turlock-Branch. The submissions came from two ranches of a company located in the Central Valley of California. The flocks had been vaccinated only against Marek's disease *in ovo*. At necropsy, the most striking findings were suggestive of septicemia, including multifocal necrotic hepatitis, splenomegaly, hypopyon, synovitis, and pericarditis. A few birds also had airsacculitis. Histopathologic examination revealed multifocal fibrinoid necrosis in the liver, lymphocytic perivascular cuffing in the brain, mononuclear and heterophilic inflammations in multiple organs including meninges, eye, joint, liver, ear, air sac, and lung, and lymphoid depletion in the bursa of Fabricius and spleen. *Ornithobacterium rhinotracheale* (ORT) was isolated from multiple organs, including the brain, liver, eye, air sacs, heart sac, spleen, trachea, and joint. One of the ORT isolated was serotype A. Infectious bursal disease virus was isolated from the bursa of Fabricius. The birds were seropositive for infectious bursal disease, infectious bronchitis, and chicken infectious anemia. ORT primarily causes respiratory disease in chickens and turkeys. However, ORT has occasionally been reported to cause septicemia and encephalitis also, although the underlying factors have not been determined.

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# HEALTH CHALLENGES OF POULTRY FARMING IN ORGANIC, ANTIBIOTIC FREE AND ALTERNATE HOUSING SYSTEMS

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**SUMMARY**

Organic poultry farming is one of the fastest growing segments of animal agriculture in the past decade in the US. This is due to a combination of increased consumer demand, environmental awareness among producers, increased antibiotic resistance of human pathogens and regulations. National organic program (NOP) is being administered by USDA. Poultry raised organically should meet animal health and welfare standards, not use hormones, not use antibiotics unless in an emergency, use organic feed, and provide birds with access to the outdoors. In spite of the growing popularity of organic farming, there are limited numbers of scientific papers published related to poultry health especially from USA. This is further hampered by lack of scientific research on raising poultry organically. Health of organically raised poultry are influenced by various factors such as breed, flock size, feed composition, rearing environment, external parasites, predators, free flying and wild birds, insects, rodents, vaccination, biosecurity, disease detection and prevention and lack of basic knowledge.

According to the NOP, "organic is a labeling term that indicates that food or other agricultural product has been produced through approved methods that integrate cultural, biological, and mechanical practices that foster cycling of resources, promote ecological balance, and conserve biodiversity" Further the Organic Standards describe the specific requirements that must be verified by USDA-accredited certifying agent before products can be labeled USDA organic. For poultry and other livestock the USDA organic seal verifies that producers met animal health and welfare standards, did not use antibiotics or growth hormones, used 100% organic feed, and provided poultry with access to the outdoors. More specifically organic meat, poultry, eggs, and dairy products come from animals that are given no pesticides, fertilizers made with synthetic ingredients or sewage sludge, and bioengineering; or ionizing radiation. Before a product can be labeled 'organic' a government approved certifier inspects the farm where the food is grown to make sure the farmer or the producer is following all the rules necessary to meet USDA organic standards. The USDA Organic seal tells that a product is at least 95 % organic. Further the USDA makes no claims that organically produced food is safer or more nutritious than conventionally produced food in the way it is grown, handles and processed.

The NOP develops the laws that regulate the creation, production, handling, labeling, trade, and enforcement of all USDA organic products. This process involves input from National Organic Standards Board (NOSB) and the public. NOSB is a Federal Advisory Committee made up of 15 members of the public. The regulations are published in seven CFR (Code of Federal Regulations) 205. Organic Foods Production Act of 1990 established the NOP and its authority to enforce agricultural products sold, labeled, or represented as "organic" within the United States.

There are also other forms of poultry being raised and sold which are appealing to the consumers for a variety of reasons. One of them is free range chicken and according to USDA the only requirement for this is to provide access to the outside. Another common practice is to raise poultry free of antibiotics called Antibiotic Free (ABF). The number of poultry flocks raised free range and ABF are increasing throughout USA. Alternate housing systems are also used in many countries in Europe. It is being implemented in California, USA effective January 2015. These alternate housing systems range from providing adequate floor space 1216 $cm^3$ in cages for each chicken to single tiered floor systems with manure bins, multi-tiered floor systems (aviaries) with litter belts and furnished cages (cages with perches, nests and dust bathing area).

Most common noninfectious disease problems reported with organic farming and alternate housing systems is increased incidence of feather picking, cannibalism and cloacal prolapse that can lead to increased mortality. Piling or smothering of chickens also occurs and contributes to the mortality. Integumentary problems such as foot pad dermatitis, hock burns, breast blisters, external parasites such as mite infestation due to *Dermanyssus gallinae* have been reported to increase in poultry raised

organically. Birds raised outdoors have a potential to be exposed to soil chemicals contaminants such as Dioxins (PCB's, PCD.s), lead, mercury, DDT, *etc*. Among the infectious agents the greatest threat to poultry raised outdoors is probably exposure to avian influenza by free flying and wild birds. Insects, rodents, and wild birds as well as predators can not only be a source of diseases but also transmit infectious diseases to poultry. Internal parasites such as Ascaridia sp., *Heterakis gallinarum*, *Capillaria* spp. and tape worms and protozoa such as coccidia and *Histomonas meleagridis* have been reported to be increased in organically raised poultry resulting in increased mortality. Among bacteria *Salmonella* spp. *E. coli*, *Pasteurella multocida*, erysipelas, *Campylobacter* spp, *Clostridium perfringens*, and even mycobacterium, etc., have also been known to cause disease in poultry raised organically but some of them can also be of great public health significance. Some of these diseases and conditions in poultry raised organically may have significant welfare implications.

It has been observed that in the past several years there is an increased incidence of gizzard erosions in broiler chickens and turkeys grown as antibiotic free or organic. There are many causes for gizzard erosions but one of the causes is probably due to the modern farming practices. There is increased pressure by the medical community to decrease the use of antibiotics as growth promotants in poultry for the fear that the use of antibiotics may result in antibiotic resistance. At the same time there is also an increased demand by the consumers for poultry grown without the use of antibiotics. Therefore, in order to keep the bacteria in low numbers poultry industry uses prebiotics, probiotics as well as various organic acids and chemicals like chlorine dioxide, hydrogen peroxide, copper sulfate, etc., in the drinking water. The organic acids and chemicals are probably safe if used in proper concentration but can result in gizzard erosions and other consequences if used in excess.

The incidence of necrotic enteritis (NE) in broilers and turkeys caused by *Clostridium perfringens* are also increasing by 100% to 200% in the last 5 years in some regions. It is probable that predisposing factors are contributing to the increased incidences of NE. But it is more likely to be related to the significant reduction in the use of antimicrobials or none at all for raising meat-type of chickens and turkeys.

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# THREE UNUSUAL OUTBREAKS OF AVIAN ENCEPHALOMYELITIS (AE) IN VACCINATED PULLETS

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## SUMMARY

Avian encephalomyelitis (AE), also called "epidemic tremor," is a viral disease of chickens, turkeys, quail, and pheasants (2,5). AE is characterized by neurological signs such as ataxia and paralysis in one to three wk old chicks and transient drop in egg production in layers. Some of the survivors can develop cataracts and can have impaired vision. AE is caused by a picornavirus and belongs to the genus *Tremovirus*. Isolates of AE are enterotropic but some show tropism to the nervous system (3,5). There are no serological differences among the isolates of AE. AE occurs worldwide. Morbidity can range from 40-60 % and mortality from 25-50 % depending on whether the chicks came from immune birds or not. Those that survive may not grow well and produce eggs normally.

The AE virus is shed in the feces during infection and it can be transmitted orally from flock to flock through various means including fomites. The virus can survive in the environment for long periods of time. Adult non-vaccinated breeder chickens, if exposed during egg production, can produce a number of eggs that may be infected and hatch as infected chicks. These chicks can shed virus and transmit the disease to hatch mates and brood mates resulting in clinical signs by seven d of age. Chicks with maternal antibodies are protected from AE. AE is rare as it has been well controlled by vaccination of breeders and pullets. A permanent immunity to AE develops within 10 to 14 d in immunologically competent chicks, i.e., after 3 to 4 wk of age. To provide maximum protection in chicks, breeding flocks should be vaccinated after eight wk of age and at least one month before egg production.

Three unusual outbreaks of AE were diagnosed in three separate flocks of chickens which had been vaccinated for AE and submitted to CAHFS by three different companies in the central valley of California. One flock was comprised of 11,500 white Leghorn 12 wk old pullets. Two other flocks were comprised of brown pullets, flocks of 80,000 and 65,000 and 11 and 11 to 14 wk of age respectively. The chickens had been vaccinated for AE and fowl pox in the wing web one or two weeks prior to the onset of clinical signs. Clinical signs included ataxia, paralysis, lateral recumbency and occasionally head tilt in 5-8 % of the chickens. Mortality and culls in the three flocks ranged from 1-3 %. Serologically most of the birds were positive for AE virus at low titers. The three flocks were negative for avian influenza and APMV-1 by PCR. Analysis of the brains for sodium and livers for heavy metals from five birds was unremarkable.

Necropsy of more than 45 chickens did not reveal any significant gross lesions. Microscopically there was disseminated non-suppurative encephalomyelitis with characteristic swollen neurons and central chromatolysis and aggregation of lymphocytes in the muscular layers of the proventriculus. In addition, other lesions such as lymphocytic neuritis, pancreatitis myocarditis, myositis, iridocyclitis and lymphocytic inflammation in the wall of the esophagus, crop and gizzard were also observed. Avian encephalomyelitis virus was confirmed in the three flocks by RT-PCR of the brain and peripheral nerves.

It is well known that if the vaccines for AE are well adapted to egg embryos and not passed through chickens orally periodically during manufacturing they will not multiply in the intestine of chickens and can become neurotropic thus causing AE (1,4). It is most likely this is what occurred in the three outbreaks described here.

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# INCLUSION OF THE GENE THAT CODIFIES FOR AVIAN INTERLEUKIN 2 INTO A LIVE RECOMBINANT VACCINE AGAINST AVIAN INFLUENZA H5 IN A NEWCASTLE DISEASE VIRUS VECTOR

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## SUMMARY

A live recombinant vaccine against avian influenza subtype H5 in a Newcastle disease virus B1 strain as back-bone vector (rNDV-B1/AIV-HA-H5) was constructed with the inclusion of the gene that codes for avian interleukin 2 (rNDV-B1/AIV-HA-H5-aIL-2) with the objective to potentially improve its protective efficacy. SPF birds were vaccinated once or twice with rNDV-B1/AIV-HA-H5 or with rNDV-B1/AIV-HA-H5-aIL-2, and later challenged against a HPAIV H5N2 or a Velogenic NDV. Results of the trial indicate that the inclusion of the gene that codes for aIL-2 interferes with the replication capacity of the live recombinant vaccine, resulting in lower immunogenicity HI values and less protection compared with that induced by the original live single-recombinant vaccine not expressing aIL-2.

## INTRODUCTION

Live recombinant vaccines against rNDV-B1/AIV-HA-H5 and rNDV-LaSota/AIV-HA-H5 have successfully been used for several years in México and China.

AIL-2 is a soluble protein produced by T-lymphocytes, involved in regulating immunity, specifically T-cell growth factor. AIL-2 has been used successfully as an immunomodulator for enhancing immunity against NDV.

The objective of this trial was to determine if the inclusion of the gene that codes for the aIL-2 in a recombinant live virus vaccine against avian influenza (H5) in a Newcastle disease virus B1 strain as back-bone vector, potentially improve its protective efficacy in terms of immunogenicity and protection.

## MATERIALS AND METHODS

**Birds.** Newly hatched SPF white leghorn chicks were obtained directly from the hatchery and housed in isolators (Controlled Isolation Systems, Inc., San Diego, CA, USA) at the CENID-Microbiología – INIFAP/SENASICA high-biosecurity facilities (BSL-3).

**Vaccines.** A first single-recombinant live virus vaccine containing the HA gene insertion of an AIV (rNDV-B1/AIV-HA-H5) was prepared to contain a viral titer of $10^{7.2}$ mean chicken embryo infective doses (CEID50)/mL. A second double-recombinant live virus vaccine containing the HA gene insertion of the AIV plus the aIL-2 gene insertion (rNDV/B1-AIV/HA/H5-aIL-2) was developed and prepared to contain the same viral titer.

The H5 HA Ectodomain was cloned from low pathogenicity AIV. The ectodomain was fused to the transmembrane and cytoplasmic tail sequences from the NDV F protein generating a Chimeric HA protein. The avian IL-2 was cloned from purified Peripheral Blood Mononuclear Cells (PBMC) of avian origin. Chimeric H5 HA was first inserted between the P and M genes of the rNDV-B1 vector, meanwhile the IL-2 gene was cloned in the intergenic region between the HN and L genes, generating in this manner a DNA infectious clone used to rescue the recombinant rNDV-B1/AI-H5-aIL-2 from tissue culture. The presence of the chimeric HA and IL-2 genes in the viral genomes was confirmed by reverse transcription–PCR and sequencing. Expression of both recombinant proteins was confirmed by Western blotting of infected Vero and Immunoperoxidase assay. Incorporation of the HA antigen into the virion was assessed by Western blotting using an anti-chicken AIV H5 polyclonal antibody. The live recombinant vaccines and the rNDV vector were titrated and kept frozen at -70°C.

**Experimental procedure**. Birds were vaccinated once or twice at 12 and 24 d of age (DA) by eye-drop with 0.03 mL ($10^{5.8}$ CEID50) of any of both vaccines. A third group remained unvaccinated as control group. At 21 d post-vaccination (PV), birds in all groups were bled and the hemagglutination inhibition (HI) test was performed for NDV and for

AIV. Then, birds were divided into two groups containing 25 birds each. One group was challenged with a Mexican velogenic viscerotropic Newcastle disease virus (VVNDV) strain Chimalhuacán (1960) using $10^{5.3}$ $CEID_{50}$ by the intramuscular route and a second group with a Mexican HPAIV (H5N2) strain A/Chicken/Queretaro/14588-19/95 (H5N2) using $10^{7.5}$ $CEID_{50}$ by the ocular route. In both cases, birds were observed for 10 d post-challenge (PC). At this time, survival birds were humanely euthanized and incinerated.

## RESULTS

For the challenge with the VVNDV, birds in the first group vaccinated once with the live single-recombinant vaccine were protected in 92% while birds vaccinated twice were protected in 100%. Birds in the second group vaccinated once with the live double-recombinant vaccine were protected in 32% while birds vaccinated twice were protected in 52%. Mortality achieved in the unvaccinated control group was 100% with an estimated mean death time of 5.4 days.

For the challenge with the HPAIV, birds in the first group vaccinated once at 10 DA with the live single-recombinant vaccine were protected in 48% while birds vaccinated twice at 10 and 24 DA were protected in 76%. Birds in the second group vaccinated once with the live double-recombinant vaccine were protected in 40% while birds vaccinated twice were protected in 60%. Mortality achieved in the unvaccinated control group was 100%, with an estimated mean death time of 7.4 days.

Serology results for NDV indicated that birds in the first group vaccinated with the live single-recombinant vaccine resulted in higher HI mean titers ($2^{2.4}$ and $2^{3.6}$ respectively for a single or a double vaccination) than birds in the second group vaccinated with the live double-recombinant vaccine ($2^{1.1}$ and $2^{1.7}$ respectively for a single or a double vaccination). For AIV, results indicated that birds in all groups were negative ($2^{0}$). Birds in the unvaccinated control group remained negative.

## DISCUSSION

Results of this trial in SPF birds vaccinated once at 1 DA or twice at 1 and 10 DA with $10^{5.8}$ CEID50 by eye-drop and later challenged at 21 d PV with a VVNDV or a HPAIV H5N2, indicates on one hand that the live single-recombined vaccine (rNDV-B1/AIV-HA-H5) induce a moderate antibody response against NDV but not against AIV, and provide partial protection to birds vaccinated once or twice against mortality with any of the challenged viruses. On the other hand and contrary to what it was believed or expected to happen, the live double-recombined vaccine (rNDV-B1/AIV-HA-H5-aIL-2) induce a lower antibody response against NDV and no response against AIV, and showed a much lower protection against mortality to both challenge viruses in birds vaccinated once or twice, meaning that the inclusion of the gene aIL-2 induces a high production of aIL-2 in vaccinated birds capable to reduce the efficacy of the vaccine.

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# RECONSTITUTION OF INFECTIOUS LARYNGOTRACHEITIS FROM A COLLECTION OF OVERLAPPING COSMID CLONES

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## SUMMARY

Overlapping cosmid and pUC19 clones whose sequences span the genome of ILTV were used in transfection experiments to reconstitute viable virus. This is the first example of the use of large DNA fragments (cosmid, fosmid, bacterial artificial chromosome and yeast artificial chromosomes) to generate an infectious clone of ILTV. This is significant since mutations can now be easily introduced within the ILTV genome using the recombination machinery of *E.coli* in order to create new vaccine strains of ILTV.

## INTRODUCTION

New more efficacious vaccines against gallid herpesvirus type 1 (GaHV-1), the causative agent of infectious laryngotracheitis (ILT), are needed. For protection against ILT, chickens have traditionally been vaccinated with live-attenuated strains that have been attenuated by either multiple passages in embryonated eggs (chicken embryo origin [CEO]) (1) or in tissue culture (tissue culture origin [TCO] (2). These vaccines are suitable for mass application, through drinking water or aerosol spray during an outbreak, but most long-lived birds (layer and breeder) have to be vaccinated with CEO or TCO multiples times (3). Once a vaccine strain has been introduced in the field, the differentiation between vaccines and field strains is difficult due to antigenic and genetic homogeneity that exists between vaccines and virulent field isolates (4). There is a need for a "marker" vaccine strain containing an engineered alteration (i.e. gene insertion or deletion) so that a DIVA (Differentiating Infected from Vaccinated Animals) strategy can be developed for ILT. To date, differentiating vaccine strains from virulent field isolates has involved restriction fragment length polymorphisms of PCR products (multilocus PCR-RFLP analyses) (5) and limited nucleotide sequence analysis. Another shortcoming of current commercial ILT vaccines is their ability to revert to virulence (6). Various studies have demonstrated that live-attenuated vaccine strains can become more virulent as a result of simple bird-to-bird passage (7). Compounding this is the fact that live ILTV vaccine strains can also establish a latent life infection (8). Consequently, latent vaccine-derived virus can reactivate so that a flock can become an unrecognized source of infection with continuous outbreaks of disease. As a result of this and the use of high-density poultry housing with infrequent environmental cleaning, there is a continuous virus reservoir (both virulent and vaccinal) in the flocks capable of evolving to new highly virulent strains (9). An ideal vaccine for ILT would be a marker strain capable of inducing long lasting herd immunity based on cell mediated immunity (Th-1 immunity) (10), incapable of vaccinal reversion, be completely safe in birds of all ages, and cost effective. In this report we describe the reconstitution of recombinant ILTV from a series of overlapping cosmid and pUC clones derived from DNA of a virulent strain of ILTV. To demonstrate the usefulness of this technology for studies of gene function, we generated a mutant lacking a redundant *pac-2* sequence. The application of the technology described her will lead to better understanding of molecular mechanism of ILTV pathogenicity with the ultimate goal of developing better vaccines.

## MATERIAL AND METHODS

**Cells and viruses.** LMH cells were grown on tissue culture dishes coated with 0.2 % gelatin and propagated in DMEM supplemented with 10 units/mL of penicillin, 10 ug/mL streptomycin and 10% FBS. Reconstituted ILTV was propagated on confluent monolayers of chicken kidney cells (11).

**Construction of recombinant DNA.** A virulent strain of ILTV within the TCO clade was used in the construction of the overlapping cosmid clones. ILTV DNA was cloned into pSuperCos according to instruction supplied by Stratagene. Ampicillin–resistant clones were obtained and restriction endonuclease profiled. An additional clone spanning the $TR_S/U_L$ junction was generated using PCR and clones into pUC19 using Gibson fusion cloning (NEB). The complete gene encoding glycoprotein B

($U_L$27) was cloned into pEGFP-NI (Clontech) using fusion cloning. In doing so the green fluorescent protein (gfp) gene was replaced with that encoding glycoprotein B. The nucleotide sequences of the cosmids were determined using Illumina MiSeq. The sequences of the $TR_S/U_L$ clones (KLO-26 and BC-114) and pgB-NI were determined using Sanger dideoxy sequencing. A map of the genomic coordinates of the cosmid and pUC19 inserts is presented in Fig.1.

**Generation of virus from cosmid and pUC 19 clones.** Cosmid DNA was purified from 500 mL cultures (LB medium plus 100ug/mL of ampicillin) using Nucleobond BAC100 (Clontech). Similarly, plasmid DNA from pUC19 recombinants was isolated using the Midiprep alkaline lysis procedure (Qiagen). To facilitate homologous recombination, the cosmid and pUC recombinants were digested with restriction endonucleases (RE) which only recognize one RE site within vector sequences. The restriction endonuclease *Nde*I was used to linearize the $TR_S/U_L$ clones KLO-26 and BC-144 and *Sfi*I was used to linearize cosmid clones pciz34, pcix12 and pci28. Because of *Sfi*I site within the $U_S$ region of ILTV, the cosmid clone pciz52 was linearized with *Pac*I. Following digestion, the DNA was extracted with phenol/chloroform, ethanol precipitated and resuspended in water. The DNA concentrations were measured using a Qubit mini fluorometer (Invitrogen) and equimolar amounts of digested cosmids and pUC recombinants (either KLO-26 or BC-114) were mixed and precipitated overnight at $4^oC$ using the calcium phosphate precipitation procedure with a glycerol boost. Confluent monolayer of LMH cell were trypsinized and 5 X $10^6$ cells were transfected with the precipitated cosmid and pUC recombinant DNA, including 2.0 ug of ancillary expression plasmids encoding ILTV $U_L$48 and ICP4. Transfected cells were incubated at $37^oC$ under 5% $CO_2$ and monitored daily for signs of cytopathic effect (CPE). CPE was observed on d five and whole-cell freezer preps were made on d six post transfection. Briefly, the medium containing cell-free ILTV was removed and store at $-80^oC$ while the infected cells were washed twice with 1 X PBS, trypsinized, pelleted and resuspended in freezer medium (DMEM containing 20%FBS and 7.5% DMSO) and store at $-80^oC$. The stability of the ILTV recombinant was investigated by sequential passage (three times) in monolayers of chicken kidney cells. For comparison purposes with viruses generated from cosmids/pUC19 recombinants, wt ILTV virus was obtained by infecting CK cells with the USDA reference strain of ILTV.

**Real time quantitative PCR.** The amount of ILTV DNA in the supernatants of infected cells was quantified by real-time PCR (qRT-PCR) in a duplex assay normalized for the host DNA (12). The real-time PCR assays were carried out on an Applied Biosystems 7500 Fast Real-Time PCR System (Life Sciences, Carlsbad, CA). The multiplex reaction contained specific primers and probes for the detection of the $U_L$44 gene of ILTV (encoding glycoprotein C) and the endogenous control gene (avian α2-collagen gene).

**Immunofluorescence assay (IFA).** Chicken kidney cells were grown in 12 well dishes and infected with either cosmid/pUC derived virus or wt ILT virus. After three d post infection these cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. A mouse monoclonal antibody specific for $U_L$44 (glycoprotein C) was used as the primary antibody (1/500 dilution) and a rabbit anti mouse fluorescein (FITC) conjugate antibody was used as the secondary at a 1/1000 dilution in the presence of vectashield (Vector Labs). Fluorescence was observed using a Zeiss AxioSkop Fluorescence Microscope (Fig. 2).

## RESULTS AND DISCUSSION

Based on whole genome sequence analysis of 18 ILTV strains it was discovered the ILTV contains an unusual number of packaging signals. All other alphaherpesviruses with D-type genomes contain only two distinct two packaging sites (*pac-1* and *pac-2*). ILTV does contain a single *pac-1* site at its 3' end, but it contains two *pac-2* sites within 1 Kb of its 5' $U_L$ region (See BC clone Fig. 3). It was reasoned that this "extra" packaging site, an inverted copy, might result in a non-viable virus following transfection due to the presence of DNA sequences essential for virus production that lie between the two sites. Because of this, two strategies were attempted to reconstitute ILT virus from a cosmid library. The first strategy involved all the overlapping cosmid clones pciz34, pcix12, pci28 and pciz52 along with a pUC19 recombinant (BC-144) containing wild type ILTV 5' sequences (two *pac-2* sites) and ancillary plasmids. The second strategy was to use all the cosmid clones plus a 5' recombinant (KLO-26) containing a deletion in the second packaging site (coordinates 831-932 on the ILTV USDA reference strain) and the ancillary plasmids.

When transfected into LMH cells each of the two cosmid/pUC19 recombinant sets generated viral plaques within 5-6 d post transfection. Thus, the problem anticipated for the set containing the BC recombinant (wild type sequence with the additional packaging site) did not materialize. The successful generation of viable reconstituted virus using the set containing the KLO-23 clone indicated that the

additional packaging site was non-essential. The absolute numbers of plaques obtained in the two individual transfection experiments involving the two sets of cosmid/pUC19 combinations depended on the amount of DNA transfected. In our initial transfection we observed CPE within five d (Fig. 2) and harvested the supernatants and infected cells for subsequent passages. Real time quantitative PCR was used to monitor the amount viral DNA in the supernatants during three passages in CK cells. Although the copies numbers were encouraging in early passages, they did not increase exponential in later passages. This low number of viral genomes in later passages and the unusual small-plaque phenotype indicate that we have generated an ILT virus with a postulated syncytial defect (Syn mutant). Clues to possible genetic defects responsible for the Syn phenotype were provided by the nucleotide sequences of the cosmid and pUC clones. Multiple alignments of the nucleotide sequences of the cosmids and pUC recombinants along with the complete genome sequences of all ILTV strains present within the GenBank data set were generated. Although various SNPs could be identified within the sequences of the cosmids, only one was not found in other ILTV strains (Fig. 4). This single mutation was present in the pciz34 cosmid within the $U_L27$ gene encoding glycoprotein B, a well characterized culprit involved in syncytia phenotypes (13). To correct for this, it was reasoned that inclusion of a plasmid containing wild type gB within the transfection set might result in the incorporation of the wild type gene within the reconstituted virus. However, this addition to the transfection set did not solve the problem and small plaques were observed even in subsequent passages. Thus the reconstituted virus was still impaired in cell-to-cell spread. Sequence analysis of the viral DNA within the supernantant of the various passages indicated that wild type gB sequences were indeed incorporated within the genome. This indicated that another gene or genes [e.g. $U_L10$ (gM) $U_L53$ (gK), $U_L20$, $U_L45$] may likely be involved in the Syn phenotype.

In conclusion, we have been able to demonstrate that viable virus can be reconstituted from overlapping cosmid clones of ILTV. Unfortunately the virus generated showed a growth impairment in comparison with wt when chicken kidney cells were infected and monitored for production of infectious virus during a six d period. It was shown that the redundant copy of the packaging site (*pac-2*) within the 5' end of the ILTV genome was non-essential for reconstitution of viable virus and the syncytia phenotype responsible for poor cell-to-cell spread involved other loci than that encoding glycoprotein B. We are currently in search of those loci involved in this genetic defect.

## ACKNOWLEDGEMENTS

We thank Walter Fuchs for the six cosmid constructs and Laszlo Zsak for helpful suggestions and advice during this study.

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**Fig. 1**. The position of the cosmid and pUC 19 inserts relative to the genome of ILTV.

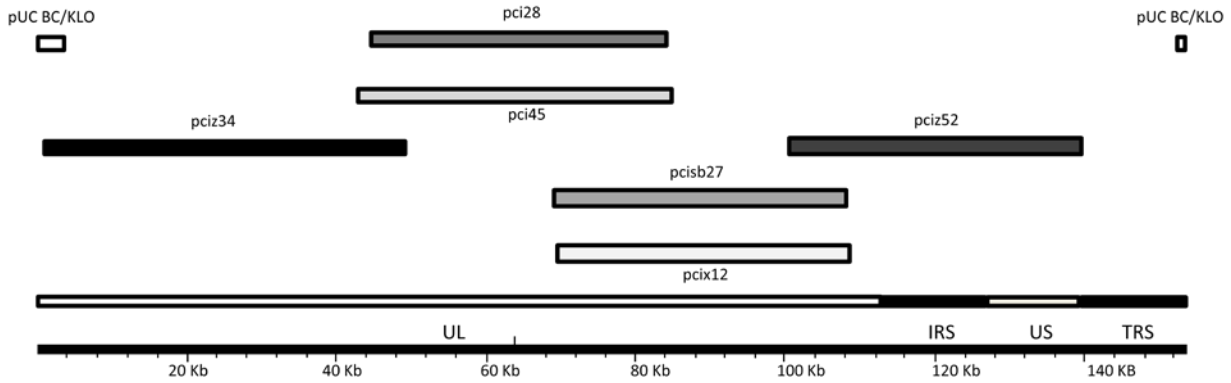



**Fig. 2**. IFA and visible microscopy of CK cells infected with reconstituted recombinant ILTV derived from collection of cosmids and pUC19 clones. Images were taken five d post infection. P1 is the first passage of the recombinants containing with the BC (wt sequences) or KLO (pac-2 deletion mutant). P3 is the third passage of the recombinants along with CK cells infected with the USDA reference strain. The small plaque phenotype of KLO is present in the lower right hand corner.

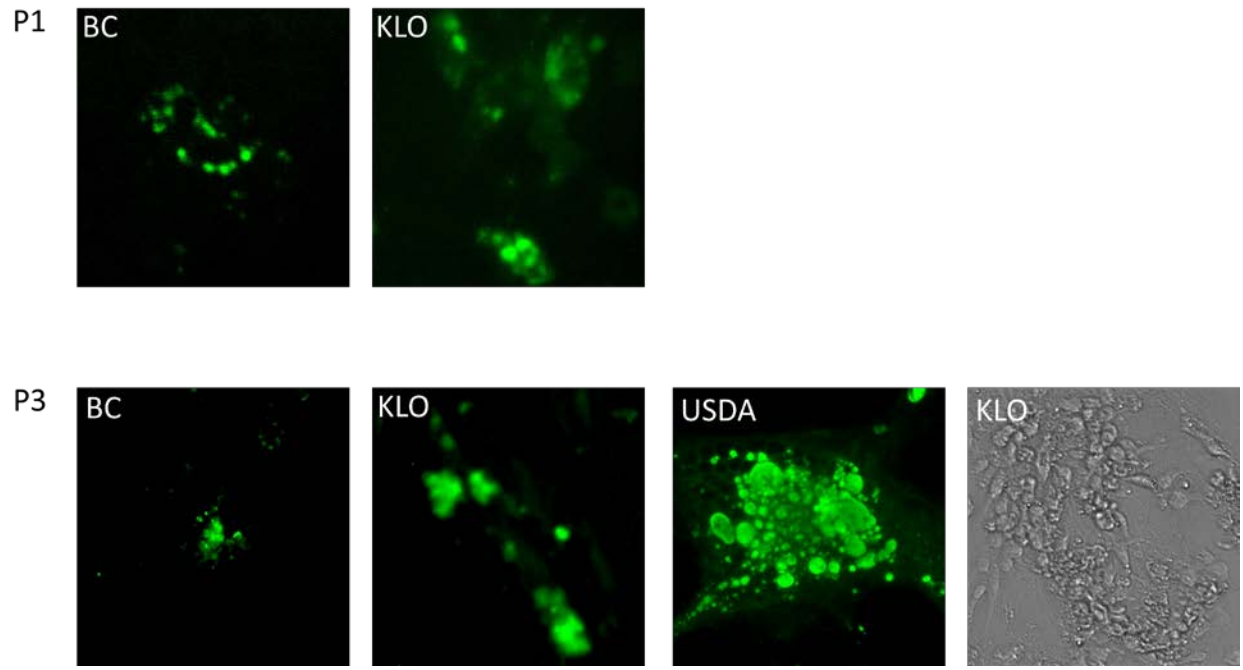


**Fig. 3**. Diagram of the two pUC19 recombinant used in this study showing the positions of the packaging sites (i.e. *pac-1* and *pac-2*) at the $TR_S$ and $U_L$ junctions. It should be noted the clone KLO-26 does not contain the second *pac-2* site.

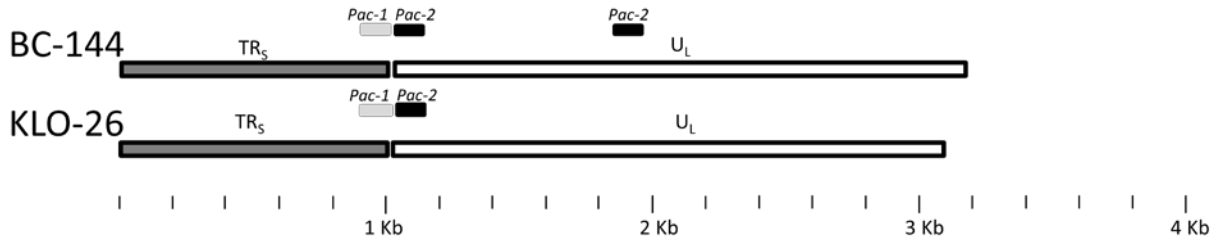
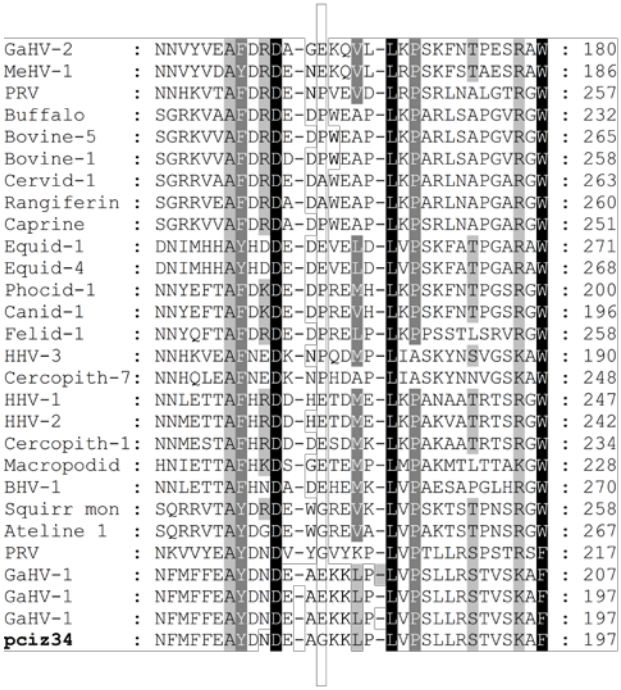



**Fig.4**. Multiple alignments of the glycoprotein B domain containing the glycine residue (G) found in the sequence of the pciz34 cosmid clone relative to those gB homologues of other alphaherpesviruses containing glutamic acid (E), proline (P), or alanine (A). All other glycoprotein B genes within ILTV (GaHV-1) contain the codons encoding glutamic acid.



# EVALUATION OF PROTECTION INDUCED BY AN INACTIVATED *RIEMERELLA ANATIPESTIFER, E. COLI* BACTERIN

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## SUMMARY

This study evaluated the duration of protection induced by a bacterin containing formalin inactivated strains of *Riemerella anatipestifer* serotype 1, 2, 5 (RA1, RA2, RA5) combined with *E. coli* 078 (RA-EC). Pekin ducks were vaccinated at two and three wk of age with 0.5 mL of RA-EC bacterin. Ducks were subsequently challenged with a virulent strain of either *E. coli* 078, *R. anatipestifer* types RA1, RA2, RA5, administered at four, six, and eight wk of age. Groups of unvaccinated controls were also challenged with a virulent strain of either *E. coli* 078, RA1, RA2, RA5 at four, six, and eight wk of age. Comparison of vaccine protective index, histopathology and antibody titers, indicate RA-EC bacterin provides protection against virulent stains of RA1, RA2, RA5 and *E. coli* O78 up to market age of ducks.

## INTRODUCTION

Acute *R. anatipestifer* infection primarily affects ducks between two to eight wk of age. To date 21 serotypes designated numerically have been identified worldwide by agglutination reactions (4). *R. anatipestifer* serotypes 1, 2 and 5 are the most common serotypes identified in commercial ducks in the United States (4,6). *R. anatipestifer* serotypes exhibit type specific agglutination with homologous antisera with the exception of serotype 5 which exhibits minor cross reaction with serotypes 2 and 9 (3,6).

A bacterin with a combination of RA1, RA2, RA5 and *Escherichia coli* O78 (RA-EC) was developed in the early 1980's (Cornell University Duck Research Laboratory, Eastport NY). The *E. coli* serotype O78 was determined to be the predominant serotype (59/100) isolated on commercial duck farms in the US in the 1980s. In field trials, the RA-EC bacterin was more effective at reducing *R. anatipestifer* mortality in White Pekins compared with a bacterin containing RA1, RA2 and RA5 without the *E. coli* component (5,7). There are no published reports indicating if immunization with RA-EC bacterin induces adequate protection that persists throughout the grow-out period in commercial Pekin ducks. Most commercial Pekin ducks are marketed between seven and eight wk of age in the US. The aim of this study was to determine if protection induced by RAC-EC immunization extends throughout the market age of ducks.

## MATERIALS AND METHODS

**Experimental ducks.** The 240 White Pekin ducks used for this experiment were from the Cornell University Duck Research Laboratory (DRL) closed, unvaccinated *R. anatipestifer* free flock. From day of age, ducks were kept in environmentally controlled, positive pressure isolation facilities for the duration of the experiment. Ducks were provided with commercial duck feed (Eastport Feeds, Inc. Eastport, NY) and water *ad libitum*. Each experimental group of 10 was kept separately in isolation facilities.

**Bacterial cultures.** All bacterial cultures used in the production of formalin inactivated RAC-EC bacterin originated from field isolates from naturally occurring disease outbreaks on commercial Pekin duck farms in the USA. The method for the RA-EC bacterin production from the TSA plated cultures, bacterin safety and protective index two wk post vaccination have been previously described (2,7). The RA-EC formalin inactivated bacterin contains approximately $10^8$ cells of *E. coli* and $10^9$ cells of RA1, RA2 and RA5 per dose (0.5 mL). Bacterial cultures used in challenge experiments were virulent strains of *E. coli* O78:K80, RA1, RA2 and RA5. Plate count titrations of harvested bacterial cultures were performed to obtain $6.5 \times 10^9$ CFU/mL *E. coli*, $4.8 \times 10^9$ CFU/mL RA1, $6.5 \times 10^9$ CFU/mL RA2, and $3.0 \times 10^9$ CFU/mL RA5 based on the previously determined median lethal dose for each strain ($LD_{50}$).

**Experimental design.** Bacterin inoculations were performed in 120 White Pekins twice at two and three wk of age with 0.5 mL of RAC-EC bacterin s.c. in the dorsal neck. The other 120 Pekins were unvaccinated and raised separately from vaccinates. Groups of 10 vaccinated and 10 unvaccinated controls were subsequently challenged with 0.5 mL

of either RA1, RA2, RA5 or *E. coli* cultures administered s.c. in the medial thigh. Challenge experiments were conducted in groups of vaccinates and controls at two, four, or six wk post immunization (four, six, and eight wk old ducks). Ducks were observed twice daily for mortality and morbidity. The vaccine Protective Index (PI) was calculated as [(% mortality in controls- %mortality in vaccinates)/(% mortality in controls)] x 100. At seven d post challenge, surviving ducks were humanely euthanized and necropsied. Heart and brain swabs were taken from each duck in each experimental group for bacteriology. Evaluation of bacterin efficacy was determined based on the vaccine protective index, re-isolation of *E. coli* or *R. anatipestifer* serotypes post challenge, an evaluation of histopathology post challenge and the serological response to vaccination using tube agglutination.

## RESULTS

All non-vaccinated controls were negative for agglutination when tested against RA1, RA2, RA5 and *E. coli* antigens at two, four, and six wk post vaccination. The tube agglutination GMT ranged from 0.4- 2.254. The GMT were higher against RA1, RA2, RA5 and *E.coli* in vaccinates at four and six wk post vaccination. The EC-RAC bacterin induced a protective index between 70-100% in ducks challenged against RA1, RA2, RA5 and *E. coli* four, six, and eight wk of age. In eight wk old ducks, the mortality induced by RA2 was not statistically different between vaccinates and controls in spite of the high protective index of 80%. In all other experiments, the mortality rate in vaccinates was statistically lower than the mortality rates in non-vaccinated controls at four, six, and eight wk of age. There was no mortality in any of the vaccinated groups challenged with either RA1, RA2, RA5 and *E. coli* at six and eight wk of age. Mortality rates ranging from 10-30% was observed in four wk old birds challenged with RA1 (30%), RA2 (10%) and *E. coli* (10%). In spite of this mortality, there was still a statistically significant drop in mortality in vaccinates compared to controls indicating that the EC-RA bacterin does have some protective effect even at four wk of age.

In the vaccinated RA1, RA2 and *E. coli* groups that died post challenge, petechial hemorrhages in the heart was the main lesion observed. *Staphylococcus aureus* was isolated from the hearts of the ducks that did not survive the challenge from the vaccinated groups. Challenge bacteria was re-isolated from all of the unvaccinated controls from RA1, RA2, RA5, *E. coli* at four, six ,and eight wk of age. Challenge bacteria were not re-isolated from the vaccinated groups except for RA2 at eight wk of age where RA2 was isolated from the heart of the one bird that did not survive challenge.

## DISCUSSION

In spite of the high protective index and an overall statistically significant decrease in mortality in vaccinated groups challenged at four, six, and eight wk of age, some low level mortality and morbidity did occur in some of the vaccinated groups. All unvaccinated control groups had more severe septicemic lesions than their vaccinated counterparts. In challenge control groups the polyserositis lesions were characterized by fibrinous pericarditis, hepatitis, airsacculitis, pneumonia and swollen mottled spleens. In the *E.coli* challenge controls, livers were generally discolored dark green. This discoloration was generally not observed in the RA challenge groups. There were no other macroscopic or microscopic lesions that facilitated differentiation of the different serotypes of RA from each other or from *E. coli*. There was complete protection (0% mortality) in all vaccinated groups challenged with RA5 at four, six, and eight wk of age. This suggests that the EC-RA bacterin is most efficacious against RA5. The EC-RA bacterin seemed to be less efficacious against RA2 since mortality in four, six, and eight wk old vaccinated groups ranged from 10-30% and the mortality rate in vaccinates vs controls was not statistically different at eight wk of age.

In spite of high levels of protection in the vaccinates, the geometric mean titers against RA1, RA2, RA5 and *E. coli* whole cell antigens was generally low at the measured post vaccine time points (four, six, and eight wk of age). This may be attributed to the functional inadequacies of duck antibodies and the lack of effectiveness of duck antibodies in precipitin and agglutination tests (1). Ducks frequently elicit a weak antibody response to antigenic stimulation from bacterial pathogens and antibody response alone cannot be used a measure of vaccine efficacy and protection. Due to the difficulties in interpretation of protection based solely on the immune response, the PI is typically used to assess the efficacy of RA vaccination.

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# INFECTIOUS BURSAL DISEASE SITUATION IN MEXICO

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ZOETIS

## SUMMARY

One major challenge for the modern poultry industry is the control of immunodepressive diseases that have a direct or indirect negative impact on poultry economics and performance. Diseases such as infectious bursal disease (IBD, Gumboro disease), chicken anemia, and mycotoxins have effects on the bursa of Fabricius, which is the main lymphoid organ of birds. Therefore, the ongoing surveillance of bursal morphological, anatomic, and functional traits is extremely important to maintain healthy birds.

Since infectious IBD was reported in Mexico, it still remains as one of the most important diseases causing economic losses. For more than ten years we have sampled the main areas of production, and through the years we have used different tools to classify the characteristics of the virus. We have used imaging processing and molecular techniques from PCR RFLP to sequence. These studies have helped us to establish the challenge and epidemiology of the virus.

We have established a bursal monitoring program that allows us to evaluate the efficacy of vaccines and vaccination programs in the control of immunodepressive diseases. The program generates parameters to assess the anatomic and physiologic integrity of the bursa from birds subjected to a vaccination program, thus generating a very useful database. The continued execution of the bursal monitoring program also allows for the comparison of results from different units or micro-regions within a company or a group of companies, leading to regional evaluations.

Early results using sequencing as a tool determined there were three types of field virus in Mexico. One was considered unique and was grouped with Delaware E virus, but in a separate branch. It had a very different amino acid sequence, but most of the field viruses were classified under the branch of variant/classic hybrid and classic ones. (Table 1)

In the last two years we have found some viruses that have a close homology to a Canadian virus. This sequence was submitted to GeneBank and found to have high sequence homology, 98.5% the IBD virus Canada (ABL85143.1). In the past year samples submitted from a different area have a highest sequence homology, 98.7-99.6%, with the IBD virus from Canada (GenBank Accession # ABL85143.1) as seen on the phylogenetic tree below. These are two different companies and are separated by more than 400 miles.

In South America since 1997 a very virulent form of IBD is present but so far has not affected Central America. In the last years a virulent form of IBD virus has been identified in the west coast of US. Therefore, it is important to continuously monitor and keep track of the virus.

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**Figure 1**. HE stain/imaging processing 41% lymphoid activity.

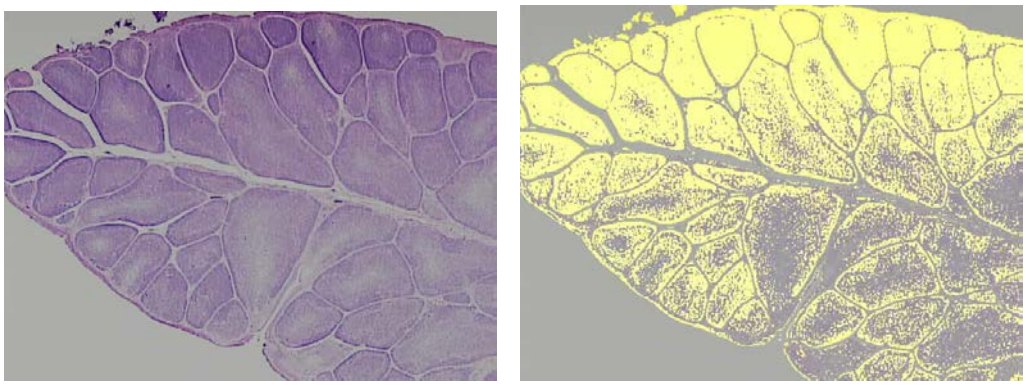

**Table 1**. Early sequencing results.

| | | | | |
|---|---|---|---|---|
| unique | T222 | K249 | I286 | D318 |
| variant classic | T222 | Q249 | T286 | D318 |
| Classic | P222 | Q249 | T286 | G318 |

# PROTECTION MEDIATED BY *IN OVO* DELIVERED TOLL-LIKE RECEPTOR 21 LIGAND AGAINST INFECTIOUS LARYNGOTRACHEITIS VIRUS INFECTION

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**SUMMARY**

Oligonucleotides containing unmethylated CpG motifs (CpG-ODN) are recognized by TLR21 receptor (homolog of TLR9 in mammals) in chickens to release pro-inflammatory cytokines. The immune protection mediated by CpG-ODN in chickens has been well reported against some bacterial and viral infections. However, its potential as an immunoprotective agent when CpG-ODN is delivered *in ovo* against infectious laryngotracheitis virus (ILTV) in post-hatch chickens has not been investigated. In this study, we investigated whether *in ovo* delivered CpG-ODN can protect the chickens by reducing the mortality and morbidity induced by post-hatch ILTV infection.

We first delivered the CpG-ODN *in ovo* into 18 embryo day (ED) eggs to make it available at the respiratory mucosa of the embryos, and incubated until they hatch. Then, we challenged the CpG ODN and control PBS treated chicks with ILTV on the d of hatch and monitored the infected birds daily for 12 d post-infection in order to determine the end points based on clinical signs and bodyweight gains. The clinical signs were scored, body weights were recorded and the tracheal swabs were taken to measure the viral infection.

We found that *in ovo* delivered CpG-ODN significantly decreases morbidity and mortality associated with post-hatch ILTV infection and decreases ILTV replication as assessed by mRNA expression of ILTV proteinase kinase gene and absolute ILTV genome load in trachea at seven d post-infection. This is the first report that *in ovo* delivered CpG-ODN has been evaluated to have immunoprotective effects against post-hatch ILTV infection in chickens.

# RAISING BIRDS WITHOUT ANTIBIOTIC FEED: WHAT IS REQUIRED? NUTRITIONAL STRATEGIES AND DIETARY ALTERNATIVES

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## SUMMARY

Well formulated broiler rations keep broiler flocks healthy, improve growth performance, and increase flock profitability. This becomes even more important for broiler flocks raised without antibiotics. Good quality feed ingredients, with highly digestible protein are essential. Diets formulated with well-balanced amino acids can be fully utilized by birds with less nitrogen from crude protein entering the caeca for fermentation, thus decreasing the chance for *Clostridium perfringens* proliferation, and minimizing necrotic enteritis. When phytase is included in the diets, more attention should be paid to other minerals, such as sodium, chloride and calcium, because they can have an impact on litter quality, flock health and performance.

Dietary non-nutritive supplements such as feed enzymes, phytogenics, probiotics, prebiotics, and other additives have been widely explored and researched as alternatives for raised without antibiotics (RWA) flocks. The responses are promising, yet not always consistent; due to dietary composition, level of background cocci challenge, and other management factors such as flock density, ventilation and litter conditions.

## INTRODUCTION

Broiler production from RWA flocks had been a niche market several years ago, but today it is trending to become a main stream product. A survey group expects US antibiotic-free broiler production will double by 2019 (36). The increase in RWA production is mainly driven by the market, or consumers' demand. However, RWA production is also supported by a decrease in the additional production costs between regular flocks and RWA flocks, largely due to genetic improvement, new technologies in nutrition, and better understanding of flock management.

There are many factors that can affect broiler flock performance ranging from nutrition and health status of breeder flocks, hatchery operations, chick quality, nutrition and water quality to flock management. To successfully grow RWA flocks, one should not only provide good management and environmental conditions as for regular broiler flocks, but should consider creating superior conditions such as reducing bird density, increasing downtime between crops, acidifying litter, and providing high quality water. Nutritionally, well balanced rations formulated with high quality ingredients are crucial for RWA flocks.

## INGREDIENTS

Wheat is one of the major energy ingredients in some parts of the world. Compared to corn, wheat contains a higher level of indigestible, water-soluble, non-starch polysaccharides (NSP). High levels of NSP in the gut will increase digesta viscosity (8), which interferes with digestive enzyme interaction with digesta and eventually impairs nutrient digestion and absorption. Furthermore, the undigested, high viscous digesta that reaches the hindgut will become a good substrate for *Clostridium perfringens* to proliferate, and develop into necrotic enteritis (2). So for RWA flocks, wheat inclusion in the ration should be severely restricted if it cannot be avoided. Alternatively, NSP enzymes should be utilized to ameliorate the effect of wheat (23).

Soybean meal (SBM) is a major protein source for RWA rations because typically RWA rations are often animal by-product free. Under-processed SBM has high level of trypsin inhibitors, which impair protein digestion and lead to wet litter and depressed performance (3). Over-cooked SBM has lower available amino acids, and poor flock performance (33). Due to the high levels of both potassium and oligosaccharides, SBM inclusion level in the first ration of broiler diets should be controlled under 30%, and other protein sources such as canola meal and corn gluten meal should be considered to provide a diversified protein supply.

DDGS is a by-product of the ethanol industry, and readily available at an attractive price. Yet, its protein quality and energy level are relatively low. Recently, Macklin and Dozier (25) reported that even with a mild case of necrotic enteritis, live

performance was more negatively affected in the birds fed 15% DDGS versus those fed no DDGS. So the inclusion rate of DDGS for RWA feeds should be carefully restricted or avoided.

## PROTEIN

For standard broiler production, increasing dietary protein (amino acids) levels will lead to a heavier, leaner bird with an improved feed conversion (5). Pesti (34) analyzed a published data set and concluded that in the overwhelming majority of studies, there is a positive response in growth and feed utilization efficiency to increasing dietary protein levels, and he further indicated that the relationships are much stronger in faster growing broiler strain birds.

It has been reported and generally understood that high dietary protein content increases *Clostridium perfringen* populations in the broiler gut (13) and is a predisposing factor for necrotic enteritis.

Parker et al. (32) studied the responses of coccidia-vaccinated broilers to different protein levels. Vaccinated birds had the best weight gain, and better FCR when they were fed diets with the highest CP levels (23%), whereas chickens fed the lowest protein had highest total oocyst shedding counts seven d after infection. Lee et al (22) investigated the effect of dietary protein levels on performance of coccidiosis vaccinated broilers following mixed-species *Eimeria* challenge, and suggested that increasing dietary protein concentrations may be one management consideration to reduce or eliminate the adverse effects on broiler performance due to vaccination during the starter period.

Since decreased protein levels to prevent overgrowth of *Clostridium perfringen* will depress the bird's growth, nutritionists should instead consider improving protein quality, and using ideal amino acid ratios to minimize the nitrogen entering the lower gut to maintain a health microflora.

## MINERAL BALANCE

For RWA broiler production, litter management is critical. If the litter moisture is too high, it will provide an ideal environment for cocci and other pathogens to proliferate. Thus, the challenge will be higher for the flock, and foot pad lesions will be increased. It is well known that mineral levels, particularly sodium, potassium and chloride have a large impact on water balance and thus affect litter quality.

The proper balance of sodium, potassium and chloride is necessary for growth, bone development and amino acid utilization, and a simplified version of Na + K – Cl to describe dietary electrolyte balance (DEB) was introduced (28). Many researchers have used this concept to investigate effect of electrolytes on broiler performance and other biological parameters. The optimum DEB is about 250 mEq/kg, but the range can be from 120 to 315 mEq/kg (6, 29). One shortcoming of the concept is that it tells the balance but not the individual mineral levels. If NaCl and/or KCl are added to a diet, the DEB will stay the same level, because on a molar basis, the added Na or K and Cl are equal. Therefore, monitoring the individual mineral concentrations is necessary too.

Depending on age, season, water quality and bedding materials, Na level should be 0.17 to 0.22% and Cl level should be in excess of sodium level by 20% (24). For non-animal protein based rations containing high SBM levels, potassium is not a concern if it is not too high.

When phytase is used in broiler rations, there is strong evidence to indicate sodium metabolism is changed. Ranvandran (35) reported phytase improved Na retention, and phytase suppliers are recommending a 0.02 to 0.03% Na uplift when phytase is included in broiler rations.

Calcium level in the diet has affected the phosphorus requirement (14) and phytase activities (37). Paiva et al. (30, 31) evaluated the effect of calcium level and phytase on broiler performance where the birds were placed on used litter from a previous flock that had presented clinical signs of NE and had been confirmed by necropsy. Broilers fed 0.6% Ca supplemented with phytase showed significant improvements in P and Ca digestibility. They concluded that higher dietary Ca (0.9% vs. 0.6%) had a negative effect on mortality associated with NE and bird performance.

In the past, it was generally considered that minerals were inexpensive and nutritionists might have included an extra safety margin for these related nutrients. Based on Paiva's research, we should rethink this concept, and decrease the levels of some minerals, particularly when phytase is used in poultry diets where mineral availability is improved.

## BETAINE

Betaine is a nutrient which can be found in sugar beets, spinach, wheat and seafood. Betaine (trimethylglycine) can be a methyl donor in the methionine homocycteine cycle, and there are reports which indicate it can spare methionine or choline. Another important characteristic of betaine is its osmolyte property.

In broiler digestion, post–meal osmotic pressure will induce movement of water from cells into the lumen of digestive tract (12). Due to the high growth

rate, today's broilers suffer more from post-meal osmotic pressure than previously. If betaine is added in the ration, it is absorbed quickly into the epithelial cells, and protects cells against osmotic stress, prevents cells from dehydration, and volume shrink. Betaine is an organic osmolyte, and increases the water retention of cells, replaces inorganic salts, and protects intracellular enzymes and protein structures.

Klasing *et al.* (20) reported that the shortening of duodenal villi due to E. Acervulina was less severe in chicks fed 0.1% betaine than in those fed an unsupplemented diet. Malabsorption of methionine was significantly reduced in E. acervulina-infected chicks following dietary supplementation with betaine (4). The recommended inclusion rate for betaine in RWA diets should be 1 to 1.5 kg/metric tonne.

## PHYTASE

Phytate exists widely in plant originated ingredients. It was reported (40) that phytate will bind protein when pH < 4.5, and chelate calcium and trace minerals when pH >4.5. In the presence of multivalent cations ($Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$), protein and phytate will form protein-mineral-phytate complexes at neutral pH (7). Yu et al (43) observed that phytate IP6 has a much stronger aggregation power with protein than IP5 and other lower molecular weight esters of phytate at pH 2.5. Phytate also inhibits pepsin activity and decreases protein digestion (43). Conwieson et al (9) reported that ingestion of phytate IP6 increased endogenous loss of nitrogen, amino acids, ion, sodium, sulphur and sialic acid.

Phytase was originally used to decrease phosphorus pollution and save feed costs. In the early days, it originated from fungus, and cleaved the phytate phosphate from the three position. Phytase recovery was a concern after the pelleting process (not heat stable) and the responses to adding phytase to poultry rations were not consistent. Additionally, there were sometimes side effects such as wet litter in broiler and turkey barns. As technology improved, phytase evolved from fungal 3-phytase to bacterial (E-coli) 6-phytase. The current generation of phytase is more efficacious, resistant to proteinase digestion, more heat stable, and widely used in animal production. At the same time, we understand how to use it better. Conwieson et al (10) studied the effect of different doses of phytase on broiler performance, and observed that the use of high dose of phytase can improve nutrient availability in poultry diets beyond that of diets containing lower phytase activity. Conwieson *et al.* (11) demonstrated that dietary supplementation with myo-inositol or phytase was effective in improving performance of commercial chickens. The higher dose of phytase will lead to higher levels of phytate destruction and provide birds with myo-inositol which will improve bird's performance.

## NSP ENZYMES

Besides phytase, there are also many other feed enzymes available, such as amylase, proteinase, and non-starch-polysacchrides (NSP) enzymes. Depending on the feed ingredients in the ration, NSP enzymes should vary accordingly. For example, xylanases should be added to wheat, rye or corn based diets, whereas glucanases should be added to barley or oat based diets (41).

These NSP enzymes can degrade soluble NSP, thus reducing intestinal digesta viscocity and improving nutrient digestibility, resulting in improved bird performance. NSP enzymes attack and break down plant cell walls, thus liberating the cell contents for other enzymes to digest, and render the long chain carbohydrates into smaller polymers or oligosaccharides. Furthermore, these short-chain oligosaccharides serve as a substrate for beneficial bacterial fermentation, just like prebiotics, and will lead to changes in the hindgut microflora. A new theory of the mode of action for NSP enzymes is related to peptide YY, which exerts a positive effect on bird performance (26). Peptide YY is a gastrointestinal hormone that can be released by the presence of volatile fatty acids in the lumen of the distal small intestine (16).

## PHYTOGENIC ADDITIVES

Phytogenic additives originated from plants, and have been used in human food and medicine for thousands of years. Generally phytogenic additives include herbs (flowering, non-woody plants), spices (herbs with an intensive smell or taste), essential oils (from steam or alcohol distillation), or extracts (oleoresins derived by non-aqueous solvents) (39). Among these phytogenic products, essential oils have received considerable attention, and their active ingredients such as carvacrol, thymol, eugenol, alicin and cinnamaldehyde, have been evaluated extensively.

Phytogenic additives may include a wide variety of plant products, their active ingredients may be different, and so, the mode of action varies. Research indicates that they have antimicrobial and antioxidant properties, stimulate appetite and enzyme secretion and immunomodulation. Recent research suggests that they can down regulate the innate immunity

function and stimulate the acquired immunity function (27).

There are many products in the market including some synthetic products with concentrated active ingredients. Many published data shown they can improve flock performance (19), but some research has found there was no response by adding these additives (17). The inconsistent results could be due to purity and concentration of the products, flock management and health condition, nutritional status.

## PROBIOTICS

Probiotics are also called direct fed microbial (DFM) in the US. The mode of action is to compete for available receptor sites and nutrients with pathogens, and produce or secrete metabolites, such as short chain fatty acids and bacterocin, thus changing the gut mocrofloa, and bird performance (15).

A good probiotic should be non-pathogenic, have a high recovery rate after high temperature pelleting, tolerate long storage time, survive the low pH in the upper digestive system, resist enzyme attack and be unaffected by bile salts environment, and proliferate quickly in the gastrointestinal tract. Thus they will be able to compete for receptor sites and produce inhibitor metabolites against other microorganisms (1).

Organisms commonly used include *Lactobacillus sp., Enterococcus sp., Bifidobacterium* spp., spore-forming *Bacillus* spp., and the yeast *Saccharomyces cerevisiae* (18). The spore forming *Bacillus* has received more attention because it can survive the pelleting process. Unlike the colonizing species *Lactobacillus*, *Bacillus* are free flowing, non-colonizing species, so they need to be administered continuously. The results of the published data are promising (21), yet, more research is required to have more consistent results.

## PREBIOTICS

Prebiotics are feed components that are not digested by host animal but selectively promote beneficial bacterial growth. In this category there are manno oliglosaccharides (MOS), fructo oliglosaccharides (FOS), lacto oliglosaccharides (LOS), and so on. There has been considerable research done to investigate the effects of these products on animal performance and health, yet the responses are quite variable (42, 18).

The mode of action can be different depending on the prebiotic. In general, they promote beneficial bacterial growth and change the microbiota environment, thus reducing the pathogen growth. MOS derived from yeast cell wall will bind type-1-fimbriae bacterial, like E. coli and salmonella (38), and reduce their chance to attach to the intestine tract.

## CONCLUSIONS

For RWA production, before investment is made into alternatives for antibiotics, nutritionists should emphasize nutritionally well-balanced diets. This starts with careful selection of dietary ingredients, which should be highly digestible and of consistent quality. Next, ideal protein concept should be utilized, and balanced mineral contents, particularly sodium and calcium should be optimized when phytase is included in the diet. Feed enzymes, such as phytase and NSP produce consistent responses, and improve flock performance. The scientific community needs to continue to investigate other alternatives, such as phytogenics, probiotics and prebiotics to elucidate their mode of action to obtain more consistent results.

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# IMMUNOMODULATORY NUTRIENTS MODIFY IMMUNE RELATED GENE EXPRESSION AND *SALMONELLA* TISSUE PRESENCE DUE TO *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS INFECTION IN CHICKENS

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## INTRODUCTION

There are many different factors that modulate the way the immune system responds to a pathogen. Immunomodulatory nutrients alter the immune response to pathogens; modulating that individual's resistance to pathogen challenges. Pathogens undergo fast-paced evolution creating problems for vaccination and antibiotic control (1), therefore it is important to investigate alternative avenues. In wild flocks of birds, different quantities and proportions of immunomodulatory nutrients will likely be consumed by each bird. This results in different individuals to have a different response to a pathogen due to their different diets. This phenomenon could diminish the rate of disease spread and be flock protective in both wild and production settings. The overall objective of this study was to characterize the immune response of layer chicks treated with known immunomodulatory nutrients: corn oil (control), fish oil, conjugated linoleic acid (CLA), and lutein, during *Salmonella* Enteritidis (SE) infection.

## MATERIALS AND METHODS

Female layer pullets (Hyline-W36) were utilized for this study (n = 96). At three wk of age birds were weighed and randomly allotted to 24 pens. This study compared four dietary treatments using six replicates pens with four chicks per pen. Isocaloric and isonitrogenous diets consisted of a rice/ soybean meal basal with supplemental corn oil (control; 3%), fish oil (3%), CLA (1%), or lutein (0.25%). After seven d of diet adaptation all birds were inoculated with 7.5 X $10^6$ CFU of SE in 1 mL PBS (verified via SE culture at 7.3 X $10^6$ CFU). On d two, four, and eigth post-inoculation one bird per pen was culled for sampling of the liver, spleen, and duodenal and ileal mucosal scrapings for rtPCR analysis. Data were analyzed using JMP (SAS Inst. Inc., Cary, NC), using a 3x4 factorial, with main effects of experimental d (two, four, or eight post-inoculation with SE) and dietary treatment (corn oil, fish oil, CLA and lutein). Overall treatment effects with a probability of $P<$ 0.05 were accepted as statistically significant using mean separation Tukey's Studentized range test to identify significance.

## RESULTS AND DISCUSSION

**Duodenal mucosa (DM): Increased Th1 immune related gene expression in lutein fed birds.** The DM is the first location where the SE challenge will likely encounter the immune defenses. In the DM there were interactions between dietary treatment and experimental day for IL-1β where D8 lutein fed birds had 50% more ($P=$ 0.03) expression than the D8 CLA fed birds; there also was an interaction for IL-12 where the D2 lutein fed birds had 0 expression, and D8 lutein fed birds had the greatest ($P=$ 0.02) amount of IL-12 expression. Lutein fed birds also had a trend for increased ($P=$ 0.08) IL-1β and increased ($P=$ 0.075) IL-12 when compared to the CLA fed birds and for IL-12 versus the corn oil fed birds. The corn oil fed birds however had a trend for increased ($P=$ 0.065) IL-6 expression when compared to the CLA fed birds. The lutein and fish oil fed birds had higher ($P<$ 0.05) anti-inflammatory IL-10 expression than the corn oil treatment. Overall, the lutein treatment had more of an inflammatory response to SE in the DM when compared to the CLA fed birds specifically. Macrophage exposure to *Salmonella* increases the expression of pro-inflammatory cytokines, including as IL-1β, IL-6, and IL-12. Both IL-1β and IL-6 can also be expressed from intestinal epithelial cells. These cytokines function to recruit phagocytic cells to the site of infection (2). Therefore, because the immune response to SE is majorly a Th1 response, there was lower ($P=$ 0.05) SE invasion, as measured by the expression of SEF14, in lutein fed birds (higher inflammatory gene expression), than the CLA fed birds (less inflammatory gene expression).

**Ileal mucosa (IM): Increase in expression of all immune response genes in lutein fed birds.** One of the major places of SE colonization is the ceca (3), therefore the IM is a major site of proliferation. In the IM the lutein fed birds had high immune related gene

expression for: IL-1β (*P*=0.021) compared to corn oil fed birds; Higher IL-8 (*P*<0.01) compared to corn oil and CLA fed birds; a trend for increased IL-6 with fish oil (*P*=0.069) compared to corn oil fed birds; increased IFN-γ (*P*= 0.027) and iNOS (*P*= 0.007) with the corn oil fed birds, compared to the CLA fed birds; increased (*P*< 0.008) anti-inflammatory IL-10 expression over the corn oil fed birds; and on d eight both the lutein and fish oil fed birds had higher IL-10 expression than the corn oil fed birds. There was a trend for increased (*P*=0.065) IL-4 expression in the fish oil fed birds when compared to the corn oil fed birds. Overall in the IM, the lutein fed birds had the most immune related gene expression (inflammatory and anti-inflammatory) and this balanced response may have been the reason for a lack of difference in SE presence (SEF14 expression) compared to the other dietary treatments. The corn and fish oil treated birds had higher SEF14 expression than the CLA fed birds. The fish oil fed birds had increased anti-inflammatory gene expression and the corn oil fed birds had low immune related gene expression compared to all other treatments overall, leading to the increase in SEF14 expression for both treatments in the IM.

**Liver: Increased α-1-glycoprotein gene expression in the lutein fed birds.** The liver is rich in immune cells, and is where the blood from the gastrointestinal tract is evaluated for pathogens (4). Fish oil fed birds had higher (*P*= 0.05) hepatic IL-6 expression than the CLA fed birds. And on d four the lutein fed birds had higher (*P*= 0.003) α-1-glycoprotein (AGP) expression than the corn or fish oil fed birds. IL-6 is one of the cytokines that stimulate the liver to produce acute phase proteins like AGP; AGP helps to neutralize the toxicity of LPS and has previously been reported to increase with SE stimulation in birds (5). All other cytokines were not significantly affected by dietary treatment, explaining a lack of statistical differences in SEF14 expression (i.e. SE presence) in the liver.

**Spleen: Increased Th1 immune related gene expression in fish oil fed birds.** The splenic response is indicative of a systemic SE infection. Splenic IL-12 expression was highest (*P*= 0.05) in the corn oil fed birds compared to the lutein fed birds, and specifically on d two the corn oil, fish oil and CLA fed birds had higher (*P*= 0.008) IL-12 expression than the lutein fed birds. Also on d two, the fish oil and CLA fed birds had higher (P= 0.02) iNOS expression than the lutein fed birds. There was also trends for IL-8 and iNOS expression to be higher (*P*= 0.078 and *P*= 0.091 respectively) in the corn oil and CLA fed birds compared to the lutein fed birds; and for the fish oil fed birds to have higher (P=0.065) IFN-γ expression than the lutein fed birds. Interleukin-12 is important in mounting a Th1 response against intracellular pathogens (6), and induces the production of iNOS in macrophages and this process is mediated by the production of IFN-γ (7). The chemokine IL-8 is produced by the host to mediate an SE-induced heterophil infiltration to the infected tissues (8). Therefore in the spleen, the decrease in the Th1 response of the lutein fed birds may have been responsible for increased (P= 0.02) SE presence (SEF14 expression) in those birds, specifically when compared to the fish oil fed birds.

## CONCLUSIONS

Overall, we can conclude that our hypothesis was correct that different immunomodulatory nutrients induce varied effects on the immune system during a SE challenge. These differing responses were tissue specific, for example lutein accentuated a protective Th1 response in the intestine but a dampened Th1 response in the spleen. These changes are reflected in lower SE presence in the intestinal mucosa but higher invasion in the spleen. Future research will examine the effects of immunomodulatory nutrients on SE spread between birds in a flock. Future experiments will also evaluate the effects of feeding different immunomodulatory diets to each bird within the same flock changes horizontal transfer.

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