PROCEEDINGS OF THE FIFTY-THIRD WESTERN POULTRY DISEASE CONFERENCE

March 7-9, 2004 Sacramento, California

WPDC SPECIAL RECOGNITION AWARD

G. YAN GHAZIKHANIAN

The Western Poultry Disease Conference is proud to present the 2004 WPDC Special Recognition Award to Dr. G. Yan Ghazikhanian.

Galestan Yan Ghazikhanian was born in 1937 in Iran. He obtained his DVM from the University of Tehran, Iran, and was presented the Silver Coin Education Medallion by the Shah of Iran for being the top ranked veterinary graduate. He received his MS and PhD in Comparative Pathology from the University of California, Davis. Under the tutelage of Dr. R. Yamamoto, Yan researched *Mycoplasma meleagridis* and *M. synoviae* in turkeys. After finishing his PhD, he worked at USDA APHIS as a director of a mobile laboratory in the 1971-72 eradication of velogenic viscerotropic Newcastle Disease in Southern California. Since 1974 he has worked at Nicholas Turkey Breeding Farms (NTBF), starting as a field and research veterinarian. Currently, Yan is Vice-President and Director of Veterinary Medicine for the company. To everyone at NTBF, he is simply known as Dr. Yan.

In his 25 years with NTBF, Yan has established himself as an expert on the diseases and management of turkeys. Many consider him to be “The Man” on just about anything dealing with turkeys. A few of the many accomplishments Yan has made while at NTBF include the eradication of *M. meleagridis*, *M. synoviae*, and *Salmonella arizonae* infections from turkey primary breeding stock. In addition, he continues to research the turkey musculoskeletal system. Yan’s work at NTBF has significantly impacted the improvement of the health and the economic production of turkeys throughout the world.

Yan travels a lot and has visited all the major turkey-producing companies in the USA. In fact, he has probably visited most of the turkey companies in Europe and the rest of the world.

Yan served as one of the program chairmen for the first WPDC/ANECA joint meeting in 1980. The meeting was held in Acapulco, Mexico, and was a tremendous success and the beginning of a wonderful relationship with ANECA and future joint meetings.

In 2001, Yan received the Golden Rooster Award in recognition as the Person of the Year from the California Poultry Federation. In 2002, Yan was recognized by the AAAP with the C.A. Bottorff Award for his significant contributions to the poultry health program in North America.

Yan and his wife Cheryl, have two children, Jenia and Stefan. Last year, Yan became a grandfather. Yan has told us that he plans to retire this year. But we hope it won’t be the last we see of him. He is highly respected and sought out for advice by his colleagues, peers and students. He has been an invaluable reference to the western region and the entire poultry industry.

SPECIAL ACKNOWLEDGMENTS

The Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions to the Conference. The financial contributions provide support for outstanding participants and to help pay for some of the costs of the Conference. Almost 40 organizations, companies and individuals have 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.

We are extremely pleased to acknowledge two contributors at the Benefactor level. They are the American Association of Avian Pathologists and Merial Select, Inc. Once again, our distinguished Patrons, Donors, Sustaining Members, and Friends of the Conference are listed on the following pages. We greatly appreciate their generosity and say thanks to them and their representatives.

Dr. Joan Jeffrey Schrader would like to express her thanks to Rhonda Roche of the UC-Davis Veterinary Medicine Teaching and Research Center for her invaluable secretarial assistance with the program, Richard Chin for his vigilance and consistent helpfulness, Dave Frame for his philosophy and experience.

Many have provided special services that contribute to the continued success of this conference. The WPDC would like to thank Helen Moriyama, Rebecca Gonzales and Courtney Hufnagle, of the Fresno branch of the California Animal Health and Food Safety Laboratory System, for their secretarial support. For this year's meeting, the WPDC has contracted Conference & Event Services, of the University of California, Davis, for providing registration and budgetary support for the conference. We would like to thank Teresa Brown and Jennifer Thayer for their work with our conference.

We thank Dr. David Frame for editing and producing another outstanding Proceedings of this meeting. Dr. Frame is indebted to Ms. Sherry Nielson, Senior Secretary of The Utah State University Turkey Research Center, for her wise suggestions, hours of proofreading, and formatting the Proceedings for publication. We express our gratitude to all authors who submitted manuscripts – especially those who followed the instructions and submitted their papers on time! We again acknowledge and thank Ominpress (Madison, WI) for the handling and printing of this year's Proceedings, and to Microsearch Corporation (Saugus, MA) for their creation of the CD-ROM for this year's meeting. Once again, we acknowledge Bruce Patrick (Graphic Communications, Brigham Young University) for the cover design, and to Dr. Rocio Crespo for designing the CD cover and label.

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54th WPDC – 2005	Joan Jeffrey	Stewart J. Ritchie		

MINUTES OF THE 52ND WPDC ANNUAL BUSINESS MEETING

Secretary-Treasurer Chin called the meeting to order on Monday, 10th March 2003, at 5:15 PM, at the Holiday Inn Capitol Plaza hotel. President Daft could not make the meeting. There were 21 people in attendance.

APPROVAL OF 51st WPDC BUSINESS MEETING MINUTES

The minutes from the 51st WPDC business meeting were reviewed and a motion was carried to approve them as printed in the Proceedings of the 52nd WPDC.

ANNOUNCEMENTS

Secretary-Treasurer Chin acknowledged all the contributors; in particular, those contributing at the Benefactor level, which included the American Association of Avian Pathologists and Merial Select, Inc. He also thanked all the contributors for their generous donations. Secretary-Treasurer Chin acknowledged the efforts of the current WPDC officers who organized the meeting.

REPORT OF THE SECRETARY-TREASURER

Dr. R.P. Chin presented the Secretary-Treasurer report. There were 195 registrants for the 51st WPDC held at the CasaMagna Marriot, Puerto Vallarta, Jalisco, Mexico, May 1-4, 2002. Contributions for the 51st WPDC were $31,850, with a total income of $79,743. There were expenses of $91,873 for WPDC for the meeting, resulting in a net loss of $12,130. The current balance in the WPDC account is $48,470.69. The loss was as expected. Most of the loss was due to the low number of registrants for WPDC as many registered through ANECA. In addition, expenses were obviously higher due to the Mexican location. The Secretary-Treasurer's report was approved.

REPORT OF THE PROCEEDINGS EDITOR

Dr. D. Frame presented the Proceedings Editor report. There were 69 papers and a total of 120 pages in this year's proceedings. For this conference, 500 hard copies and 500 electronic copies of the Proceedings were produced. The CD's cost approximately $3.00 each and the books cost $4.50 each. The books were produced by omnipress and the CD's were produced by Microsearch Corporation. The production of the CD's were much easier this year as pdf file were created and sent rather than a camera-ready copy. As usual, there were the routine headaches of trying to get people to meet the submission deadline, formatting of tables and following the author instructions.

OLD BUSINESS

There was no old business.

NEW BUSINESS

Seeing that WPDC lost money, it was suggested that we increase registration fees $5-10. It was left to the Executive Committee to look at the final budget for this year's meeting and then decide if an increase is necessary.

One person commented that they were displeased with the impromptu meeting on vaccination for exotic Newcastle disease that occurred concurrently with the afternoon session. Most people who attended that meeting felt it was very worthwhile. One such person said that WPDC needed to be flexible to account for such meetings, as there was an excellent exchange of knowledge and ideas. However, those who stayed to listen to the scheduled meeting and those speaking felt it was a disruption to the general meeting as many people attended the impromptu meeting rather than the scheduled meeting. It was felt that panel discussions could be scheduled into the talks to allow for this type of discuss and exchange of knowledge. In addition, it was suggested that scheduled breaks could be used to extend good discussions.

Dr. Shivaprasad commented that the ACPV workshop on Saturday was a success. He thanked all the speakers and the WPDC Executive Committee for help in organizing the meeting.

Secretary-Treasurer Chin reported that the WPDC Executive Committee nominated Dr. Steward Ritchie (aka Chicken Stew) for Program Chair-elect of the 54th WPDC in 2005. This was seconded. A motion was made and seconded to close nominations. Dr. Ritchie was elected unanimously as program chair-elect. Secretary-Treasurer. Chin nominated the following officers for 2003-2004:

Program Chair: Dr. Joan Jeffrey
President: Dr. David Willoughby
Local Arrangement Coordinator: Dr. Carol Cardona
Contributions Chair: Dr. Ken Takeshita
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Rich Chin
Program Chair-elect: Dr. Stewart Ritchie

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

It was announced that the 54th WPDC would be held in Vancouver, British Columbia, Canada, on April 23-26, 2005. Dr. Rosenwald still questioned the location and recommended we go to Banff. He and Dr. Ritchie were to get together after the business meeting to discuss the location.

Secretary-Treasurer Chin passed the presidency to Dr. Dave Willoughby who thanked those involved in the organization of the meeting, and adjourned the meeting at 6:00 PM.

TABLE OF CONTENTS

Note: Both the oral and poster presentations of the 53rd WPDC are listed below in alphabetical arrangement by presenter. Authors and titles of all papers scheduled for presentation at the conference are listed.

ANGULO, F. Impacts of Antimicrobial Growth Promoter Termination in Denmark 16

ANNETT, C. B. Effect of Diet on Alpha Toxin Production by *Clostridium Perfringens* Type A 84

AVELLANEDA, G. The Impact of Growth-Promoting Antibiotics on Poultry Bacterial Communities 20

BAUER, C. R. Characteristics of Capsid Sequence in Pigeon Circovirus 85

BELLI, S. I. The Molecular Basis of the Poultry Coccidiosis Vaccine, CoxAbic® 68

BOULIANNE, M. Epidemiology of *Campylobacter* and *Salmonella* in Chicken Production in Quebec, Canada 4

BURNS, K. E. Efficacy of a *H. paragallinarum* Bacterin in a Novel Oil Emulsion 79

CARDONA, C. Evaluation of immunization of Commercial Laying Hens with a Killed AI Vaccine 56

CATTOLI, G. Increased Resistance of Vaccinated Turkeys to Experimental Infection with an H7N3 Low Pathogenicity Avian Influenza Virus 60

CERVANTES, H. The Debate about Antibiotic Use in Food Animals: The Science vs. the Politics 20

CHARLTON, B. R. Turkey Respiratory Disease: Etiologic Occurrence in Diagnostic Submissions 73

CHERRY, T. Coccidial Lesions in Commercial Flocks Vaccinated with a Coccidiosis Vaccine 85

COOKSON, K. Efficacy of a Live *S. typhimurium* Vaccine Given by Different Routes and the Influence of Same-Day Antibiotic Administration 49

CORTES, P. Botulism in Pheasants, Chukars and Quails 52

COX, T. Use of Risk Assessment Models in Regulating Food Animal Antibiotics 10

CRESPO, R. Pododermatitis in Meat Turkeys 86

CRUZ-COY, J. Immunogenicity of a Fowlpox Vectored AI Vaccine 62

DAWE, J. The Relationship between Poultry Health and Food Safety 24

DHILLON, S. Broiler Chicks Inoculated Experimentally with *C. jejuni* 43

ECKROADE, R. Update on the Development of a Proposed Plan for the Control of H5/H7 Avian Influenza 53

FRIEND, T. Contemporary Poultry Housing in Germany and England 79

GALLAZZI, D. Footpad Dermatitis and Welfare in Italian Broiler Chickens 87

GARCIA, A. Consideration to Control Avian Influenza 88

GRIMES, T. — Australian Initiatives to Control Antibiotic Resistance Issues in the Poultry Industry 30

GUARD-BOULDIN, J. — Subpopulation Behavior of Egg-Contaminating *Salmonella enterica* serovar Enteridtidis as Defined by LPS Mass 44

HAFEZ, H. M. — Investigations on Detection of *Histomonas meleagridis* DNA Using PCR 71

HALVORSON, D. — Poultry Disease Control: We Can Do Better 75

HAMMARLUND, M. — Veterinary Diagnostic Service and Village Animal Health Workers in Cambodia 51

HAUCK, R. — Investigation of Serological Response to Avian REV after Experimental Infection with Fowlpox Virus carrying REV Sequences 40

JACKSON, C. — The Role of Vaccination in the Eradication of Virulent ND in Australia Particularly in Relation to the Egg Industry 65

KAPCZYNSKI, D. — Vaccine Protection in Chickens and Turkeys Against the 2002 END Virus 64

KEHLER, H. — Comparison of Real-Time RT-PCR, Conventional RT-PCR and Virus Isolation Assays for detecting AIV in Experimentally Infected Chickens and Turkeys 89

KELLY, B. — Review of Clonal Relationships between *Campylobacters* Isolated from Poultry and Humans in North America 41

LEONG, V. — Avian Influenza Control and Monitoring in Hong Kong 57

MALO, A. — Broad Spectrum Protection after the Use of an Inactivated Vaccine Containing *S. enteritidis* and *S. typhimurium* Strains Produced under Iron-Restriction 90

MARTIN, A. — Coccidiosis Immunity Development after *In ovo* Delivery of *Eimeria* Parasitic Stages 70

MC CARTY, J. — The Effect of *In ovo* IBDV Vaccination when Administered in Maternal Antibody-Positive Chickens 38

MONDAL, S. — Comparison of Four Regions in the Replicase Gene of Heterologous IBV Strain 74

MOSCOSO, H. — Molecular Identification of Avian RNA Viruses Stored on FTA Filter Paper 91

MUNOZ, R. — New FlockChek* Avian Pneuomovirus Antibody Test Kit 92

NEWELL, D. — *Campylobacters* in Poultry: Epidemiology, Ecology and the Potential for Control Up to the Point of Slaughter 1

PERELMAN, B. — Large Scale Use of a Recombinant VP2 Vaccine for the Vaccination and Control of IBD in Chickens 37

RAMIREZ, H. — Diagnosis Assistance System for Major Clinical Poultry Diseases 93

SERRATOS, A. — Genotype/Enviroment Interaction in a Commercial Line of Laying Hens 94

SHANE, S. — Evaluating Investment in Enhanced Biosecurity for Egg Production Operations 76

SHIVAPRASAD, H. L. — Avian Influenza H6N2 in California Broiler Chickens and Turkeys – 2002 63

SOMMER, F. — *Bordetella Avium*: An Opportunistic Pathogen in Chickens? 72

SONG, Y. — First Functional Assessment of a Chicken Caspase 96

SORIANO, E. — Immunogenic and Cross-Protection Studies of the Nine Serovars of *H. paragallinarum* 80

SPENCER, L. — Antibody Production and Persistence of MDV in Vaccinated Chickens 81

STERN, N. — Potential for On-Farm Control of *Campylobacter* in Broilers 8

TAKESHITA, K. — Survival of Exotic NDV in Commercial Poultry Environment Following Removal of Infected Chickens 64

TERRINGO, C. — Evaluation of the Efficacy of Vaccination against Newcastle Disease in Canary Birds (*Serinus Canarius*) 96

TORO, H. — Chicken Anemia Virus: Oral Versus Intramuscular Route of Infection 39

VAUGHN, M. — Is There Human Health Harm Following FQ Use in Poultry? 27

VAZQUEZ, M. E. — Biochemical and 16S rRNA Sequencing Characterization of *Pasteurella haemolytica*-Like Organisms in Commercial Layer Flocks in Mexico 97

VILLEGAS, P. — Clinical and Molecular Characteristics of IBDV's Identified in Latin America 32

WALLNER-PENDLETON, E. — Game Bird Health and Management Education in Pennsylvania: Past and Present 51

WESTALL, S. — Investigation into the Cause of Excessive Early Mortality in Broilers 50

WHITEFORD, A./ SHEER J. — California Experience with Exotic Newcastle Disease: A State and Federal Regulatory Perspective 81

WOOLCOCK, P. — Viruses Associated with Poult Enteritis in Turkeys Grown in California 1993-2003 98

ZANELLA, A. — IBD: Field Evaluation on Effects of Two Vaccines with Different Levels of Residual Pathogenicity in Broilers 34

PROCEEDINGS OF THE FIFTY-THIRD WESTERN POULTRY DISEASE CONFERENCE

CAMPYLOBACTERS IN POULTRY: EPIDEMIOLOGY, ECOLOGY AND THE POTENTIAL FOR CONTROL UP TO THE POINT OF SLAUGHTER

Diane G. Newell

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ABSTRACT

Campylobacter jejuni and *C. coli* are common bacterial commensals of the avian gut but are major food-borne causes of human acute enteritis. Reduction of the risk to human health from *Campylobacter* contaminated poultry is a priority in many countries. Unfortunately, the biosecurity measures currently used for broilers have been largely ineffective for *Campylobacters*. However, molecular epidemiological studies are now beginning to accurately identify the sources of those *Campylobacters* colonizing flocks. The evidence to date suggests that intervention measures, at least initially, need to be targeted at horizontal transmission from the external broiler house environment.

INTRODUCTION

Campylobacter jejuni, and its close relative *C. coli*, are Gram-negative, highly motile, microaerophilic and thermophilic bacteria, now recognised as major causes of human acute bacterial enteritis world-wide (1). *C. jejuni* in particular is ubiquitous in the environment and colonizes the gastrointestinal tracts of most mammals and birds. Although there are multiple potential sources of infection for man, epidemiological studies implicate the consumption or handling of raw or undercooked poultry meat as a major route.

C.jejuni/coli commonly colonize the intestinal tracts of most poultry at slaughter including broilers, laying hens, ducks, turkeys, and game birds (2). This colonization is generally asymptomatic. Although hepatitis in chickens, associated with *Campylobacters* recoverable from the liver, has been reported, the disease is not experimentally reproducible, and, therefore, these bacteria are unlikely to be the cause.

The epidemiology of *Campylobacter* colonization in broiler flocks, though well investigated, remains unclear (3). For the majority of flocks, colonization is not detectable until the birds are two to three weeks of age. In the initial stages of flock colonization the within-flock prevalence can be low but shedding is rapid and bird-to-bird transmission, even within flocks of penned birds (4), is so efficient that within two to three days up to 100% of birds become colonized. Thus, once the first bird becomes colonized then infection of the whole flock seems unavoidable. This is because *Campylobacters* are remarkably efficient colonizers of the avian gut. Cecal levels of up to 10^9 cfu per gram of cecal contents are common. Consequently the potential bacterial pathogen load entering a poultry abattoir with each infected flock can be as high as 10^{12} cfu. The subsequent fecal contamination of poultry meat products during processing is inevitable, thereby constituting a significant risk to human health as confirmed by quantitative risk analysis (5).

Controlling *Campylobacters* in the food chain is now a primary objective of many food safety authorities. However, the development and implementation of intervention strategies requires knowledge of the extent of the problem. Structured national surveys are difficult and expensive to undertake in such a complex and fragmented industry. Nevertheless, ongoing surveillance in some European countries indicates that the prevalence of broiler flock colonization at slaughter varies between countries, and with season and management practices. In countries like Denmark, the Netherlands, and Great Britain the overall prevalence is about 50%; but in northern European countries, such as Sweden, this prevalence is down to about 10% (6). Prevalence usually peaks in the summer months and is dependent on the type of production system, for example reaching 100 % in organic and free-range flocks (7). Variation in prevalence between national flocks may be, at least in part, a consequence of different husbandry or environmental factors but may also be a reflection of differences in sampling frames and detection methodologies. Standard detection procedures are urgently needed and in support of the new European Zoonoses Directive (2003), a collaborative group is currently establishing what the minimum requirements for national surveillance should be.

Many potential intervention strategies at the farm level have been proposed (3). In general these either aim to prevent the birds becoming colonized with *Campylobacters* or to modify the gut environment thereby reducing the extent of colonization. The latter strategy includes measures such as vaccination, probiotic treatments and the breeding of genetic

resistance. Such measures generally remain in early research phases. The remainder of this review will focus on measures to identify and control the potential sources of flock colonization.

Sources of broiler flock colonization and associated control measures. The possible sources of *Campylobacter* colonization in poultry have recently been reviewed (3). All available data suggest that the general measures taken to control *Salmonellae* in poultry have little, if any, effect on the prevalence of *Campylobacter* colonization in flocks. This is presumably a reflection of the considerable differences in ecology and physiology between these two organisms and suggests that a more targeted approach to the identification and subsequent control of potential *Campylobacter* sources is required. The development and use of molecular typing tools for *Campylobacters* has begun to enable such approaches to be adopted (8). The problem of *Campylobacter* genetic instability, which has generally hampered the use of such techniques to study the epidemiology of human campylobacteriosis (9), appears to be far less important in the acute outbreak events associated with poultry flock colonization, provided that a layered strategy for typing is adopted. However, unfortunately, *campylobacters* in and around the broiler house environment tend to be difficult to recover and maintain *in vitro*, which to date has constrained available data. A combination of random sampling and molecular typing procedures has been developed to attempt to overcome some of these problems. In this approach the flock within, and the surrounding environment of, individual broiler houses are sampled from chick placement until the flock becomes positive. The environmental samples are enriched for *Campylobacter* recovery and initial growth then stored frozen. The strain from the flock is isolated and the short variable region of the *flaA* gene sequenced so that an oligonucleotide sequence can be designed unique to the colonizing strain. This is used to generate a labelled probe for incorporation into a lightcycler assay (10). The assay is then used to survey all the environmental samples to determine the presence of potential sources of that specific strain. This novel approach is now allowing the retrospective identification of the potential environmental sources of those strains colonizing the broiler flock.

In such molecular epidemiological investigations, most broiler flocks in Europe are colonized by only a limited number (1-2) of strains suggesting a point source outbreak (6, 11). The role of vertical transmission as a potential source is debatable. Organisms are recoverable from the urogential tracts of laying hens (12, 13), and even from the semen of cockerels (14), and by PCR *Campylobacter* DNA is associated with newly hatched chicks (15). Nevertheless, on the basis of the presence of the lag phase, the lack of recoverable organisms from chicks and lack of similarity between strains colonizing parent and broiler flocks, vertical transmission is generally considered a relatively unimportant route of flock colonization (3).

In contrast, horizontal transmission appears to have a major role. These organisms are ubiquitous in rural environments, where they are regularly shed from most domestic and wild animals and birds and can contaminate soil, concrete, equipment, and surface water. In addition, broiler house environments, internal and external, are heavily contaminated when colonized flocks are in residence. Survival, but not growth, of *Campylobacters* in such environments can occur for months especially in temperate, moist, and dark conditions. However, because these organisms survive poorly in dry conditions, poultry feedstuffs and fresh litter have been largely eliminated as potential sources.

Molecular epidemiological investigations in European broiler houses that are routinely cleared between flocks, suggest that house cleansing and disinfection is generally adequate, even after occupation with a colonized flock, (16) and consequently carry-over from one flock to a subsequent flock in the same house is relatively infrequent (17). Largely by elimination this suggests that *Campylobacters* from contaminated external environments are the major source. This is generally supported by typing studies demonstrating that strains recovered from environmental samples, like wild birds feces and puddles, can be recovered from subsequently colonized flocks (18, 19). The major route of transmission of such environmental organisms into a house appears to be via farm staff. Interestingly, flock thinning (the planned partial depopulation of a flock) (20), with associated increases in human traffic, is a significant risk factor for flock positivity.

Other significant risk factors indicated in at least some epidemiological studies include unchlorinated water supplies, the presence of vermin or dung beetles, and other livestock on the same site (3).

Finally, even if a flock remains *Campylobacter*-negative at slaughter age, the crates in which the birds are collected, and subsequently transported to the abattoir, are demonstrably contaminated with *Campylobacters*, despite washing (21, 22) and the strains on these crates, prior to loading, have been recovered subsequently on the carcasses of the birds (21).

Many measures have been recommended to reduce the risk of flock colonization (23) including:

- All-in all out-policy;
- Remove litter between flocks and disinfect buildings between flocks

- Maintain buildings in good repair and with intact concrete apron
- Clean water and/or effective water treatment
- Dispose of dead birds properly
- Change and disinfect boots
- Change outer protective clothing
- Avoid thinning during production cycle
- Restrict visitor access
- Do not keep other domestic animals on the same site

However, to date in experimental intervention studies such measures only delay, rather than prevent, the onset of colonization (24). Thus it seems likely that biosecurity alone will be insufficient to consistently produce negative flocks and that complementary measures, such as vaccination or probiotics, will be required.

CONCLUSIONS

Worldwide, much of the poultry meat presented at retail is contaminated with *C. jejuni* and/or *C. coli*. This contamination is largely a result of the preferential colonization of the avian tract by these organisms. The role of poultry-associated strains in human infection remains to be established, but there is clearly a need for the control and prevention of *Campylobacters* in poultry and poultry meat products. In the future this will require the development of innovative and sequential intervention strategies throughout the food production chain. In the meantime, improved knowledge of the biology of the organism and its interaction with its various hosts are essential. The increasing availability of *Campylobacter* genome sequence data, and subsequent post-genomics studies, will hopefully enable rapid progress towards targeted approaches for intervention.

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EPIDEMIOLOGY OF *CAMPYLOBACTER* AND *SALMONELLA* IN CHICKEN PRODUCTION IN QUEBEC, CANADA

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SUMMARY

A prospective observational study was conducted to estimate prevalence and identify risk factors associated with chicken carcass and cecal contamination with *Salmonella* spp. and *Campylobacter* spp. Fifty-eight broiler chicken flocks were sampled in Quebec, Canada, between April and December 2003. Data were gathered from single bird examination, laboratory analysis, questionnaires and condemnation sheets. A total of 87.5% and 69.0% of tested lots were positive to presence of *Salmonella* spp. and *Campylobacter* spp. respectively on carcasses, while 50.9% and 28.1% killing lots were found to harbor *Salmonella* spp. and *Campylobacter* spp. respectively in their ceca. Risk of cross-contamination at the plant between lots with *Campylobacter* spp. was found to be of lesser importance than with *Salmonella* spp.

Poultry meat is often incriminated as a common source of food borne pathogens. *Salmonella* has long been associated with poultry products but in the last decade, attention has turned to *Campylobacter* as an important cause of food borne bacterial gastroenteritis in humans. In parallel, the poultry processing industry has seen the implementation of HACCP procedures in order to decrease bacterial carcass contamination. Numerous studies have reported a wide range of prevalence for both *Salmonella* and *Campylobacter*, this being probably caused by various protocols and sampling sites used.

Both bacteria are commonly present in clinically healthy chicken gastrointestinal tract and are found on poultry products after processing. Fecal contamination of skin and feathers during transportation, leakage from the crop or the cloaca, intestinal breakage and contact with contaminated equipment, water or other carcasses during the evisceration process have been cited as possible sources of bacterial contamination. Since fecal carcass contamination has often been related to improper feed withdrawal, optimal feed withdrawal times corresponding to a low fecal content have been estimated (12). None of these studies has ever looked at the effect of feed withdrawal and intestinal emptying in relation to bacterial carcass contamination.

Identification of control measures requires a good understanding of the epidemiology of both bacteria in poultry meat production. While a great deal is known about *Salmonella* means of transmission, there are still debates as to how *Campylobacter* spp. is mostly transmitted. Our knowledge of the epidemiology of *Salmonella* has helped in establishing various and

effective preventive measures at every level of production; hatchery, farm, feedmill, *Salmonella*-free parent and grand-parent flocks, use of vaccination, competitive exclusion, etc…, which finally seem to be succeeding in reducing levels of *Salmonella* in broilers in countries where implemented (2). Unfortunately, similar measures appear to be ineffective against *Campylobacter*. Although it has been suggested that vertical transmission may play a role in the epidemiology of *Campylobacter* infections in broiler flocks (13), it is usually agreed that horizontal transmission from the environment into broiler houses (3, 8) might be the major route of flock colonization for *Campylobacter* spp. Finally, simple biosecurity measures have been shown to delay flock colonization (1, 4, 6), but did not avoid it.

Objectives of our study were therefore to 1) estimate prevalence, 2) evaluate the association between carcass and cecal contamination at lot level for each bacteria, 3) evaluate the association between *Salmonella* and *Campylobacter* cecal contamination at lot level, and 4) identify risk factors at bird and flock level associated with chicken cecal and carcass contamination with *Salmonella* spp. and *Campylobacter* spp.

MATERIALS AND METHODS

Study design. A prospective observational study was performed in the province of Quebec, Canada, between April and December 2003. This study contained three sampling hierarchal levels i.e., slaughterhouses, broiler flocks, and chickens. Data were collected from the Canadian Food Inspection Agency condemnation sheets, Environment Canada database, questionnaires, single bird examination, and laboratory analysis.

Slaughter houses, broiler flocks, trucks and chickens selection. Broiler chicken carcasses were obtained from four major slaughterhouses in the province of Quebec, Canada. One slaughterhouse per week was randomly selected within a month period for a total of 10 visits to each slaughterhouse. For each visit, two broiler flocks, consecutive when possible, were randomly selected based upon producer's acceptance and time of slaughter (between 7h00 and 14h00). Broilers were selected from a single truck in order to increase precision related to transportation data, for a total of thirty broiler chicken carcasses per truck (killing lot) being systematically sampled.

Single bird examination. Thirty broiler chicken carcasses, and matching digestive tracts, were sampled from the process line, after evisceration, and individually examined for presence and type of visual contamination, sex, body weight and intestine characteristics (emptying, aspect, enteritis). Intestines were brought on ice to the lab, frozen and stored at –70°C to be later measured for their tensile strength.

Questionnaires. Producers answered a questionnaire which covered husbandry (housing, equipment, light, litter, feed, water, vaccines, medication, growth promoters, anticoccidial program, mortality, feed and water withdrawal, loading) and biosecurity measures (rodent control, cleaning/ disinfection, farm site, visitors) during downtime and the rearing period. A second questionnaire was answered by the quality control manager at slaughter house and provided information regarding birds transportation, birds cleanliness upon arrival, and slaughtering process. Questionnaires were sent by mail or fax within two days following slaughter. Meteorological data for the growing period and time of transportation were also obtained from Environnement Canada.

Bacteriological methods. Sampling. A carcass rinse was performed on each sampled carcass right before visual examination as described by Line *et al*., 2001 (9). Corresponding intestines were collected directly from the eviscerating line, ceca were sampled, individually packed in sterile plastic bags, and stored on ice up to 8 h until being processed in the laboratory. Cecal analysis was performed on three pools of ten ceca each (in duplicate; one for *Salmonella* spp., the other for *Campylobacter* spp. isolation), after aseptic cecal content collection.

Culture and identification of *Campylobacter*. Carcass rinse was analysed by adding 25 mL to an equal volume of double-strength Bolton Broth (BB). The broth preparations were incubated at 37°C for 24h before 10 μL portions were plated onto charcoal cefaperazone desoxycholate agar (CCDA) plates and incubated microaerobically at 37°C for 48h. The identities of presumptive *Campylobacter* isolates were confirmed by cell morphology, oxidase activity and mobility. For cecal analysis, direct plating was done on CCDA agar and presumptive isolates were confirmed as previously described.

Culture and identification of *Salmonella.* BPW-carcass rinses were incubated for 24h at 37°C and were then incubated for selective enrichment at 37°C in Rappaport-Vassiliadis (RV) and Tetrathionate Brilliant Green Broth (TBG). After 24h, a 10 μL loop was streaked from each selective enrichment broths onto Brilliant Green Agar (BGS) and modified lysine iron agar (DMLIA) supplemented with novobiocin (20μg/mL). These plates were incubated for 24h and 48h at 37°C. Presumptive colonies were confirmed by biochemically using urea and triple sugar iron (TSI) and were tested with polyvalent antisera for O and H antigen (Official method-Mega-rule, FDA, USDA, 1996).

Statistical methods. Confidence limits (CL, α=0.05) for the various prevalences were calculated using the Binomial exact test. Strengths of association between carcass and cecal contamination, and *Salmonella* and *Campylobacter* cecal contamination, at lot level, were calculated with the Chi-Square exact test (presence or absence of contamination) or the median test (proportion of contaminated carcasses). Risk factors associated with carcass contamination, using the bird as the statistical unit, were evaluated in a univariate analysis using generalized linear models with logit link function and exchangeable correlation matrix for chickens within flocks. The GENMOD procedure in SAS System 8.02 was used. Parameters were estimated by generalized estimating equations (GEEs) with empirical standard error estimates, and *P* values were calculated using the standard normal distribution. Risk factors associated with the presence of cecal contamination at lot level were evaluated using the Chi-Square exact test.

RESULTS

Data presented in the present paper were obtained from 58 broiler chicken flocks sampled between April and December 2003. A lot was considered positive for carcase contamination when there was at least a single isolation for each one of the studied microorganism, and positive for cecal contamination when the bacteria was isolated from at least one of the three cecal content pools. For the purpose of this presentation, only four dependant variables (presence or absence of *Salmonella* spp. and *Campylobacter* spp., in ceca and on carcass) and twenty-two independent variables were selected and categorized for statistical analysis and discussion.

A total of 88% (CL:76-95) and 69% (CL:56-81) of tested lots were positive to presence of *Salmonella* spp. and *Campylobacter* spp. respectively on carcasses. Carcass contamination distributions are shown in fig. 1. A total of 51% (CL:37-65) and 28% (CL:17-42) killing lots were found to harbor *Salmonella* spp. and *Campylobacter* spp. respectively in their ceca.

An association was demonstrated between the proportion of contaminated carcasses in a lot and the cecal status. For lots with *Campylobacter*-positive ceca, 88% of the carcasses on median were positive in the lot relative to 3% for *Campylobacter*-negative ceca lots (P<0.001). For *Salmonella,* the median of positive carcasses was 37% within ceca-positive lots compared to 9% for ceca-negative lots (P<0.01). The presence of carcass contamination in a lot was also associated with the cecal status of the lot for both bacteria ($P<0.05$, results not presented). A positive association (P=0.03) was also observed between cecal contamination for *Salmonella* and *Campylobacter*. For lots with *Salmonella*-positive ceca, 39.3% were ceca-positive to *Campylobacter* spp., while only 11.5% of the lots with *Salmonella*-negative ceca harbored *Campylobacter*.

Risk factors tested for carcass contamination at bird level included jejunal and ileal aspect, content, and presence of enteritis, and single bird body weight deviation from the average killed lot body weight. Heavier broilers have a tendency to have more carcasses contaminated with *Salmonella* (P=0.09), whereas lighter birds appeared protected against *Campylobacter* contamination (P=0.03). No intestinal parameters were associated with carcass contamination. The analysis of risk factors at flock and slaughter levels is in current process.

Risk factors tested for the cecal contamination were the average killed lot body weight, total condemnation (%), hatchery of origin, litter appearance and flock density (kg/m^2) at the end of the rearing period, downtime duration, rodent control method used, total number of birds raised per year on the same production site, presence of animal proteins in the diet, total feed withdrawal time before slaughter, time without feed on the farm, time in transportation cages, total transit time from the farm to the slaughter house, holding time at slaughter. A humid litter at the end of the rearing period was associated with an increased risk of *Campylobacter* cecal contamination (P=0.03). Producers using professional rodent control services had broiler flocks more at risk to have ceca contaminated with *Salmonella* (P=0.02) or *Campylobacter* (P=0.07).

DISCUSSION

Our objectives were to estimate prevalence, evaluate the association between carcass and cecal contamination at lot level for each bacteria, and the association between *Salmonella* and *Campylobacter* cecal contamination at lot level, and finally to identify risk factors at bird and flock level associated with chicken cecal and carcass contamination with *Salmonella* spp. and *Campylobacter* spp.

Prevalence of *Campylobacter* spp. observed in our study are similar to those reported in a previous study (11) and by other authors (6, 7). While prevalence for *Salmonella* appears to be high, it is important to note that a lot was considered positive if a single carcass tested positive. Furthermore, our carcass contamination distribution show that the majority of *Salmonella*-positive lots (41%) had less than 20% *Salmonella*-positive carcasses, and that only 5% of the positive lots had more than 80% *Salmonella*-positive carcasses. This infectious pattern within a lot was entirely different for *Campylobacter* where prevalence was lower. Indeed, 25% of the *Campylobacter*-positive lots had a large proportion (>81%) of contaminated

carcasses. This finding was also observed in our previous study (11) and is probably related to the rapid spread within a flock once the bacteria is introduced (3, 8).

The nature of the poultry processing system makes cross-contamination almost unavoidable but the level of *Campylobacter* cross-contamination was of much lesser importance than initially expected. Indeed, if 56.1% lots showed at least one contaminated carcass with *Campylobacter*-negative ceca, proportion of contaminated carcasses within such a lot was only 3,3%.

Intestinal appearance was not associated with bacteria carcass contamination. Since we did not evaluate crop content and that organ has been incriminated in carcass contamination (5), slaughter house feed withdrawal policies should probably mostly ensure that crops are properly emptied prior to slaughter. Further analysis should reveal if intestinal strength can be related to carcass contamination.

A humid litter at the end of the rearing period was associated with an increased risk of *Campylobacter* cecal contamination (P=0.03). This finding supports the role of litter in the perpetuation and transmission of this bacteria (10). To our surprise, professional rodent control was a risk factor associated with greater risk of cecal contamination with *Salmonella* (P=0.02) or *Campylobacter* (P=0.07). This could only be explained by possible cross-contamination from technicians servicing other farms and species (3, 14).

In conclusion, we found that risk of cross-contamination at the plant between lots with *Campylobacter* spp. was found to be of lesser importance than with *Salmonella* spp.

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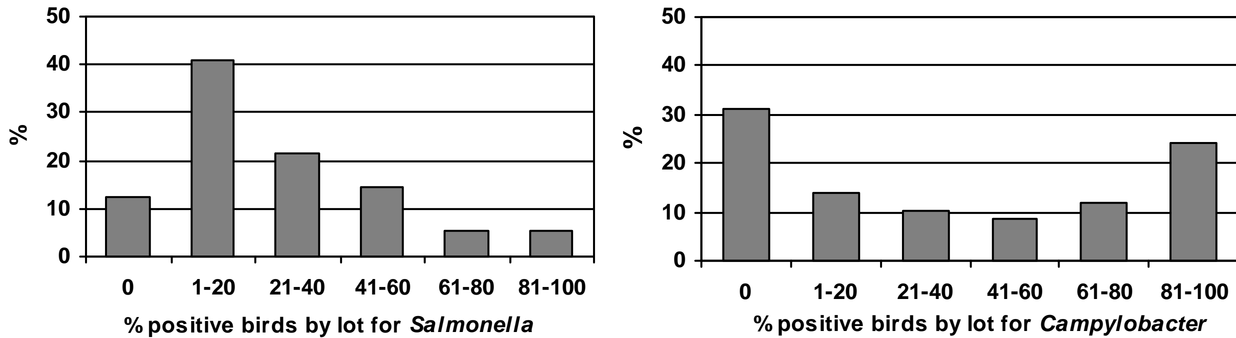
Figure 1. Carcass contamination distribution for *Salmonella* spp. and *Campylobacter* spp. *Salmonella* spp. (n= 56 lots); mean proportion =26%, maximum=93%, *Campylobacter* spp. (n=58); mean proportion=39.5%, maximum=100.0%.



POTENTIAL FOR ON-FARM CONTROL OF *CAMPYLOBACTER* IN BROILERS

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ABSTRACT

Campylobacter jejuni is an important food borne agent of human gastroenteritis. This organism is thought to be transmitted most frequently through exposure to poultry products. We screened 365 *Bacillus/Paenibacillus* spp. isolates from poultry production to identify potentials for anti-*C. jejuni* activity. Zones of *C. jejuni* inhibition surrounding 56 isolates caught our interest. One novel antagonistic *Bacillus circulans* (NRRL B-30644) and two *Paenibacillus polymyxa* (NRRL B-30507 & NRRL B-30509) strains were identified and deposited under provisions of the Budapest Treaty. The cell-free, ammonium sulfate precipitate from each candidate culture also created zones of *C. jejuni* inhibition in spot tests. Exposure of the crude antimicrobial preparation to protease enzymes inactivated *Campylobacter* inhibition, thus demonstrating a peptide characteristic consistent with bacteriocin definition. The peptides were characterized by SDS-PAGE electrophoresis, isoelectric focusing, and amino acid sequencing. In 15 separate experiments, one or two days-post-hatch chicks were colonized with challenges of ~2 X 10^6 cfu *C. jejuni*, and placed in isolation units. Three days before sampling, therapeutic feeds were provided ad-libitum. This feed consisted of purified bacteriocin (0.25 or 0.5 g) micro-encapsulated in polyvinyl-pyrrolidone and incorporated into 1 Kg of chicken feed. Therapeutic treatment consistently reduced *C. jejuni* colonization by at least 100,000 fold over the untreated chicks. Therapeutic bacteriocin treatment of mature chickens prior to slaughter may substantially reduce public exposure to this organism.

RESULTS

Three hundred sixty-five strains of *Bacillus/Paenibacillus* spp. derived from poultry production facilities in Russia were screened for antagonism to *Campylobacter jejuni*. Promising isolates were selected for further analyses, including "spot tests", protein purifications, protease susceptibility, and temperature and pH stability. Isoelectrofocusing and SDS-PAGE were used to characterize bacillocins inhibitory to *Campylobacter jejuni*. The amino acid sequences were determined for the most promising of these bacillocins. Molecular weights ranged from 3,214 kDa to 3,864 kDa and the bacteriocins contained from 30 to 39 amino acids, with isoelectric points of pI 4.8, pI of 7.2 and pI of 7.8, depending upon the bacillocin. *Paenibacillus polymyxa*, designated NRRL B-30507 (# 37), NRRL B-30508 (# 119), and NRRL B-30509 (# 602); and *Bacillus circulans* designated NRRL B-30644 (# 1580) have been deposited under the provisions of the Budapest Treaty.

In 15 replicate chicken experiments, employing birds from 7 to 24 days of age, colonization by *Campylobacter jejuni* was consistently reduced among groups therapeutically treated with bacillocin # 602. Reduction in carriage levels of *Campylobacter jejuni* among the therapeutically treated chickens, as compare to the non-treated control groups of chickens, ranged from 5 to 8 log10 CFU/gram of cecal content. Therapeutic treatment of poultry flocks with bacteriocins three days before processing may dramatically control carcass contamination and reduce public exposure to *Campylobacter jejuni*.

DISCUSSION

Bacteriocins are lethal to the target organism, are effective in the mucosal surface, are effective against antibiotic resistant target bacteria, leave no residues, create no resistant target bacteria and are produced cheaply. Competitive exclusion had been attributed to several purported mechanisms: substrate competition, colonization-site competition, volatile fatty acid production, rapid rates of proliferation, and bacteriocin production. Our data indicate that bacteriocin production is most important. Bacteriocins can be selectively inhibitory to food borne pathogens.

Bacteriocins are produced in bacterial ribosomes and must be modified before becoming active. Various sub-classes of bacteriocins are described in the literature. Antibiotics are secondary metabolites and are prohibited to be incorporated into foods. Class IIa Bacteriocins are short chain (<40 amino acids in length) proteins and are susceptible to proteolysis. No residues can be excreted from the treated host. Bacteriocin modes of action are distinguished from clinical antibiotics. These bacteriocins attack susceptible host cell surfaces and cause cytoplasmic leakage and subsequent death. Bacterial resistance to antibiotics *do not* confer bacteriocin resistance. The immunity of the cell synthesizing the bacteriocin to its product is a phenomenon that further distinguishes bacteriocins from antibiotics.

Bacteriocin resistance must be further studied. The effect on normal flora should be studied, although it is unimportant if used only at the end of poultry production. The mode of action for each bacteriocin will be useful to discriminate in application. In 1988, FDA confirmed GRAS status of nisin in foods. If bacteriocins are good enough for humans, why would it not be good enough for poultry? Bacteriocins have been consumed for centuries as products of lactic acid bacteria and manifest no toxicity. Further toxicology studies needed for our bacteriocins. Our bacteriocins have been chemically characterized and can be consistently reproduced, as compared with CE flora.

The efficacy of the bacteriocins has been consistently reproduced. Studies of acute subchronic-toxicity, chronic-toxicity, reproductive issues, sensitization, and cross-resistance are needed. Preliminary tissue culture studies indicate no toxicity from our purified bacteriocins. These same types of studies were done in 1962 for nisin. Nisin is rapidly inactivated in the GI tract. Pediocin injected into mice and rabbits produced no immunologic response (Bhunia *et al*., 1990).

CONCLUSION

A novel therapeutic treatment, employing oral administration of bacteriocins to provide at least a 5 log reduction in levels of *Campylobacter* in broilers, was described.

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USE OF RISK ASSESSMENT MODELS IN REGULATING FOOD ANIMAL ANTIBIOTICS

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SUMMARY

There is widespread concern that continued use of poultry antibiotics might increase risk of antibiotic-resistant bacterial illnesses in human patients. However, insufficient use of animal antibiotics may increase pathogen loads in retail meats, human illnesses, patients-per-year treated with antibiotics, and hence emergence of antibiotic resistance among humans. Risk models are needed to quantify these competing risks and identify risk management policies to protect human health. Such models reveal that a highly effective way to reduce human health risks may be to continue to use antibiotics in poultry to prevent human illnesses and to avoid the need to treat human patients with antibiotics.

INTRODUCTION

A common regulatory concern in the US and worldwide is that continued use of fluoroquinolones, macrolides, streptogramins, and other antibiotics in poultry and other food animals might increase the risk of antibiotic-resistant bacterial illnesses, especially campylobacteriosis, salmonellosis, and streptogramin-resistant, vancomycin-resistant *E. faecium* infections in human patients with compromised immune systems (13). A less frequently assessed threat is that insufficient use of animal antibiotics may lead to increased microbial loads in food animal products and increased human illnesses, resulting in increased need to treat patients with antibiotics and hence more rapid spread of antibiotic resistance in human populations. To balance such conflicting concerns, quantitative risk models are essential. This paper reviews methods and results of quantitative human health risk assessment modeling for animal antibiotics. It discusses when such model results can be trusted and used to build confidence in and improve the quality of regulatory decisions by increasing the probability of desired health outcomes.

HEALTH RISK ASSESSMENT FRAMEWORK

Health risk assessment estimates the health risks to individuals, groups (e.g., old, young, or immuno-compromised), and entire populations from exposures to hazards and from decisions or activities that create them. Health risks describe the probabilities and magnitudes (or frequencies and severities) of adverse health effects caused by exposures. Individual risks may be expressed in units of expected adverse health effects per capita-year. Population risks are found by summing individual risks over all individuals in the population and are usually expressed in units of expected cases per year in different illness severity categories, e.g., mild, moderate, severe, and fatal (1).

Following the National Academy of Sciences, the US FDA, CDC and USDA defined risk assessment as a process that "consists of the following steps: hazard identification, exposure assessment, hazard characterization (dose-response), and risk characterization" (http://www.foodsafety.gov/~dms/lmriskgl.html). Dose-response assessment is defined as "The determination of the relationship between the magnitude of exposure and the magnitude and/or frequency of adverse effects." The main goal of risk assessment is to produce information to improve risk management decisions by identifying causal relations between alternative risk management decisions and their probable total human health consequences (including health benefits, if any, as well as risks) and by identifying those decisions that make preferred outcomes more likely. Unlike informal expert decision analysis, quantitative health risk assessment uses explicit analytic (e.g., biomathematical, statistical, or simulation) models of the causal relations between actions and their probable health effects. It applies specialized models and methods to quantify likely exposures and the frequencies and severities of their resulting health consequences.

Health risk management applies principles for choosing among alternative decision alternatives or actions that affect exposures, health risks, or their consequences. Risk management is often viewed as a process that takes scientific information obtained from risk assessment as input and that recommends choices of risk management actions as output. Health risk communication characterizes and presents information about health risks and uncertainties to decision-makers and stakeholders. Useful risk assessment and risk communication support effective risk management decision-making by providing the scientific information needed to compare alternative risk

management interventions in terms of their probable impacts on exposures and the frequency and severity of resulting adverse health effects.

The primary purpose of health risk assessments is to support improved risk management decision-making. By definition, "better" risk management decisions are more likely to produce preferred consequences, e.g., fewer illnesses, mortalities, illness-days, and treatment failures per person-year. Health risk analysis also provides a framework for rational deliberation, conflict resolution, policy-making, and international harmonization about the human health risks of commercial activities. It provides a framework for predicting how such activities interact with consumer behaviors and physician behaviors in determining the frequencies and magnitudes of adverse health outcomes.

HEALTH RISK ASSESSMENT MODELING

Traditional health risk assessment modeling includes the following steps:

- Scope the assessment to support decisions by estimating the causal relation between decisions, exposures, and their probable total human health consequences. A successful risk assessment must evaluate proposed solutions, not problems or situations. It should show the estimated frequencies and magnitudes (and uncertainties) of human heath consequences caused by different proposed risk management decisions. It is important to identify an adequate range of risk management options to assure that dominating alternatives are not overlooked.
- Hazard identification. This step uses data to provide evidence of a possible causal relation between exposures (to drug residues, microbial loads, etc.) and probable adverse human health responses;
- Exposure assessment presents data-based estimates of the frequency and magnitudes of individual exposures in a human population, for each risk management option evaluated.
- Exposure-response modeling or dose-response modeling quantifies the causal relation (if any) between levels of exposure and probability of specific adverse human health consequences for individuals with various characteristics or risk factors.
- Risk characterization integrates exposure assessment and exposure-response models and presents their implications for the frequency and magnitude of exposure-related adverse health effects in the exposed population. Total health consequences of a risk management action are found by summing impacts on human exposures to bacteria (both resistant and susceptible) over significant pathways, e.g., different foods and venues (e.g., home-cooked meals, restaurant dining, etc.) and applying exposure-response and consequence models to the changed exposures.
- Uncertainty characterization, addresses uncertainty, variability, and sensitivities in the estimated exposure-response relation for the exposed population. Uncertainty characterization should address both model uncertainties and data uncertainties. Variability analysis should address the extent of inter-individual heterogeneity in risks, e.g., due to differences in other risk factors and covariates.

This traditional framework, developed largely in the context of chemical carcinogen risk assessment, has been adapted for animal drug residues, food-borne microbial risks, antimicrobial resistant bacteria risks, and many other food safety issues. Substantial biological (e.g., genotyping), biostatistical, simulation, modeling, and sensitivity-uncertainty analysis methods have been developed to support each step.

Correctly used, the traditional framework can help select regulations that improve human health outcomes while also helping to clarify and resolve conflicts among stakeholders via analysis-deliberation. A well-conducted risk analysis enables stakeholders to participate more effectively in risk management deliberations and to communicate questions and concerns more clearly and concisely than would otherwise be possible. It does so by providing the relevant information needed to determine probable consequences of proposed actions; by showing how sensitive these predicted consequences are to specific uncertainties and assumptions in the analysis; and by communicating clearly and enabling effective participation. To these ends, it is best to avoid vague, meaningless, or subjective labels and descriptions of risk and instead to provide quantitative data-based risk estimates and uncertainty estimates where possible.

Three main types of risk assessment models are commonly used, as follows.

- Descriptive risk assessment models answer key factual questions such as: How large is the risk? What are its major causes? Who is most affected? What adverse health effects are caused by exposure to a hazard (e.g., *Campylobacter* in undercooked chicken), and what are the clinical consequences of antibiotic resistance? How sure are we about the answers to these questions, and what

would it take to reduce uncertainty enough so that recommended decisions might change?

- Predictive risk assessment models support risk management decisions by answering what-if questions that link risk management actions to their probable consequences, especially: How will human health risks change if different alternative risk management decisions or actions are taken? The actions considered typically include changes in animal antibiotic drug use, but predictive risk models can also be used to assess the probable human health impacts of changes in HACCP measures, physician prescription practices, restaurant cooking and food preparation practices, and consumer behaviors. Predictive risk assessment models should include expressions of uncertainty and identify the expected value of additional information in improving decisions.
- Prescriptive risk assessment models, also called risk management decision support models, help risk managers to make trade-offs and identify interventions or combinations of interventions that are expected to minimize total human health risk and maximize net human health benefits. Prescriptive decision-support models typically must sum projected human health impacts over multiple causal pathways (e.g., from multiple affected bacteria, food commodities, and at-risk populations, taking into account co-resistance and cross-resistance) to show the full human health consequences of different risk management interventions.

In all three types of models, a common causal template is that risk management actions change current exposures to a potentially harmful agent (the "hazard") received by individuals in a susceptible exposed population. Changes in exposures, in turn, lead to changes in expected illness rates and hence to changes in adverse health consequences of illnesses (e.g., illness-days or early deaths per capita-year) in the exposed population. Risk management options that decrease adverse health consequences are desired. This conceptual template for modeling the causal relation between exposures (and risk management acts that affect them) and probable adverse human health effects may be diagrammed as follows:

act → Δexposures → Δresponse → Δ**consequences** → ΔQALYs
↑ ↑ ↑
[behavior → susceptibility → treatment]

In this diagram, the deltas (Δ) indicate changes resulting from the action at the beginning of the chain. The quantities that can be changed by a risk management intervention ("**act**") include exposures (e.g., contaminated servings ingested per year, perhaps subdivided by amount of contamination); adverse responses (such as illnesses per capita-year); and population risk [a probability distribution of adverse health consequences, such as illness-days by severity category, per year in the population. These health consequences may optionally be aggregated into quality-adjusted life years (QALYs), if desired, to obtain a singe aggregate measure of impact.] In such a causal graph, each quantity is modeled as a random variable whose probability distribution depends only on the values of the variables that point into it. For example, the frequency of adverse responses in a population typically depends on exposures and on individual susceptibility. This frequency can be affected by risk management acts that change exposures to microbial loads in ingested foods. The conditional probability distribution of human health **consequences** given any choice of risk management **act** can be calculated via well-developed algorithms for causal graphs (3, 6, 14).

Alternatively, the expected number of adverse human health consequences per year in each severity category can be estimated more simply, to a useful first approximation, as the product of two main factors: an **exposure factor** giving the expected number of contaminated meals ingested per year; and a **consequence factor** giving the expected number of illness-days (or QALYs lost) by severity category from each contaminated meal ingested. If enough data are available, different degrees of contamination may be distinguished, and the product risk = exposure factor x consequence factor can then be calculated and summed for multiple contamination categories. More generally, this "risk = exposures-per-year x clinical-consequences-per-exposure" framework can be applied to each type of exposure (e.g., from susceptible, resistant, and cross-resistant bacteria) and each distinct at-risk sub-population and health consequence of interest, and resulting QALYs lost per year can be summed. Finally, depending on the available data sources, the exposure and consequence factors can be further decomposed into products of sub-factors. For example, the risk model:

Risk = (change in animal drug use) x (exposure factor) x (unit risk factor) x (consequence factor)

May be useful when an exposure factor giving the ratio of contaminated-servings-per-year ingested to fraction of flocks treated with an antibiotic can be estimated and when a unit risk factor (giving probability of illness per contaminated serving ingested) and a separate consequence factor (giving

expected QALYs lost per illness) can be distinguished and estimated from data. As usual, such risks must be summed over multiple paths (i.e., drugs, bacteria, sub-populations, and distinct health consequences) that transmit effects of changes in animal drug use to human health consequences.

The traditional risk assessment stages of hazard identification, exposure assessment, dose-response modeling, and risk characterization for a risk management intervention fill in the above diagram with specific hazards, exposures, and consequence variables (for hazard identification); quantify the input-output relations for the causal links (act $\rightarrow$ Δexposures) for exposure modeling, (Δexposures $\rightarrow$ Δresponse) for dose-response modeling, and (Δresponse $\rightarrow$ Δconsequences) for consequence modeling. Risk characterization specifies the changes in human health consequences caused by a specific exposure change that is, in turn, caused or prevented by a risk management intervention.

DATA SOURCES, METHODS, AND RESULTS OF RISK ASSESSMENT MODELING

The risk assessment framework outlined above has been implemented using various modeling strategies to obtain and organize the required data. Farm-to-fork models model the changes in microbial loads flowing from farm animals through transportation, slaughter, processing, storage, wholesale and retail, preparation, and cooking. Insufficient data and excessive combinatorial complexity of possible changes usually defeat attempts to simulate confidently and accurately the physical details of processes and changes in microbial loads at each stage – a source of occasional confusion and frustration to newcomers to the field. However, Monte-Carlo based statistical conditioning, which estimates the conditional frequency distribution of microbial loads leaving each stage by conditioning on the load leaving the closest previous stage for which data are available, provides a sound, practical alternative to detailed simulation of physical processes and changes. It makes unnecessary any attempts to model in the absence of relevant data, instead taking advantage of available microbiological sampling data. Farm-to-fork models may be combined with population dynamics models (8) to study the probable impacts of exposures to food borne bacteria on susceptible and resistant bacterial illnesses in human populations.

As a complement to farm-to-fork modeling, it is often more practical and useful to carry out risk assessment using what might be called clinic-to-farm modeling. This starts with a total number of adverse health consequences per year and apportions it into fractions that are estimated to be caused by various sources, specifically including any animal antibiotic uses of interest. The fraction of illnesses per year that could be prevented by different risk management interventions and the clinical consequences of such a change are estimated and used to evaluate alternative risk management options. An advantage of this approach is that it can often exploit available genotyping and microbiological data as well as epidemiological data to estimate the exposure and consequence factors needed to quantify risk.

To illustrate applied risk assessment modeling methods, consider the problem of quantifying the human health impact of withdrawing a specific animal antibiotic now in use, such as enrofloxacin, virginiamycin, or tylosin from use in chicken. For simplicity, this illustration will focus on a *pro forma* analysis of campylobacteriosis risks ignoring possible direct effects of a withdrawal of some antibiotics, such as virginiamycin, on increasing microbial loads of *Salmonella* (5). A basic quantitative risk estimate of the human health risks from withdrawing a specific drug with human-use analogs from use in chickens can be conducted using the following clinic-to-farm template:

Preventable individual risk due to resistance in *Campylobacter* caused by animal antibiotic use = (total campylobacteriosis cases reported per 100,000 people per year) x (fraction of all cases that are treated) x (fraction of treated cases that receive the human drug of interest) x (assumed treatment failure rate per resistant case treated with the human drug of interest) x (excess QALYS lost or illness-days caused per case of treatment failure) x (average true treated cases per reported treated case) x (fraction of treated cases with resistance caused by ingestion of contaminated servings of the food product of interest) x (fraction of contaminated servings in the food product of interest that would disappear if the animal drug use ceased)

Plausible order-of-magnitude numbers for these factors and for resulting human health benefits (i.e. risk reductions) from withdrawing a fluoroquinolone or macrolide drug can be calculated roughly as follows: (13.4 campylobacteriosis cases reported per 100,000 people-year (2)) x (0.006 fraction of all cases that are treated (1)) x (0.5 fraction of treated cases that receive the human drug of interest (order of magnitude estimate)) x (1%-100% assumed treatment failures from resistance per resistant case treated with the human drug of interest (12)) x (0-2 excess days of severe illness per treatment failure) x (2-32 average true treated cases per reported treated case (9)) x (0.02-0.5 fraction of treated resistant cases caused by ingestion of contaminated servings of the food product of interest) x (0.01-1.00 preventable resistance fraction of contaminated servings in the food product of interest) = (13.4/100000)*0.006*0.5*0.1*1*8*0.1*0.1

≈ **3 x 10^{-9} cases per capita-year.** The corresponding population risk is (290M people in US) x (3E-9) ≈ **0.9 excess cases and illness-days per year.**

Note: This point estimate of 3E-9 is based on the geometric means of the uncertainty intervals shown, except for the 0-2 excess illness-day interval, for which a point estimate of 1 day is used. (The lower bound of 0 on this parameter reflects the fact that no clinical adverse effects of treating a nominally "resistant" campylobacteriosis case with typical therapeutic doses has been convincingly demonstrated for macrolides or fluoroquinolones, although current physician prescription guidelines may call for a change to another drug in a day or two if resistance is encountered.) The uncertainty intervals shown may be interpreted as approximate geometric 95% confidence intervals and combined (via a central limit theorem for log-normal distributions of networks of conditional probabilities (7)) to obtain an overall uncertainty factor of 10^(2*((.5*log10(10))^2 + (.5*log10(5))^2 + (.5*log10(10))^2 + (.5*log10(8))^2)^0.5) ≈ 66-fold. This may be interpreted as an approximate geometric 95% confidence factor, i.e., the true risk is likely to be within a factor of about 66 of the point estimate based on these uncertainty intervals.

Similarly, human health benefits from continued use of the drug can be estimated as follows:

Human illnesses prevented per year by continued animal drug use = (fraction of currently healthy animals that would be replaced by ill ones, e.g., affected by airsacculitis (AS) or necrotic enteritis, if the animal drug were withdrawn) x (ratio of microbial load in processed meat from ill compared to healthy animals) x (ratio of human illness risk per cfu ingested for servings from high microbial load (e.g., ill) compared to low microbial load (e.g., healthy) animals) x (fraction of current human illnesses caused by servings from healthy animals) x (number of current illnesses per year) = (0.005 assumed fraction of animals currently given drug to prevent illness and that would become ill without it, despite use of other drugs) x (10-fold increase in microbial load at processing (11)) x (4-fold increase in risk per cfu at high doses, estimated using a log-exponential exposure-variability modeling approach suggested by CVM for *Campylobacter* (www.fda.gov/cvm/antimicrobial/RRAIntro.pdf) and from epidemiological data on chicken-associated campylobacteriosis cases) x (0.02-0.5 fraction of current human illnesses caused by servings from healthy animals) x [(13.4 reported cases per 100,000 people per year in 2002) x (290M people in the US) x (2-32 average true treated cases per reported treated case(9))] x (at least 2 average illness-days per case) = 0.005*10 * 4 * 0.1 * (13.4/100000) * 290000000 * 8 * 2 ≈ **12,400 excess illness-days per year** from withdrawal.

For purposes of this illustration, detailed derivations of the specific numbers, uncertainty ranges, and uncertainty analysis are not provided, but they should be included in formal quantitative risk assessments for any specific animal antibiotic. The main results suggested by these rough order-of-magnitude calculations are that individual risks are small and population risk (= about 290 million individuals in the US x average individual risk of 3E-9 ≈ 0.9 cases and illness-days per year) are on the order of 1 excess severe illness-day per year in the US; whereas the human health benefits of continued use (or, equivalently, the human health risks from withdrawal) of the animal drug are about four orders of magnitude larger.

A full risk assessment could also consider the timing of impacts (e.g., what fraction of the total human health benefits and risks of withdrawal are achieved within 5 years?), any additional benefits or risks due to impacts (on cross-resistant bacteria as well as *Campylobacter*) of animal drug withdrawal and resulting changes in physician and veterinary prescription practices; and longer-term impacts of withdrawal on the population dynamics of food borne illnesses, drug use, and emergence of resistance. In detailed risk assessments carried out for specific animal drugs, these factors attenuate the potential human health benefit from withdrawal, decreasing the estimated preventable risk significantly below 1 excess illness-day per year in the US population (4), while increasing estimated human health risks.

CRITERIA FOR USEFUL RISK ASSESSMENT MODELS

To be most useful and appropriate for guiding regulatory decision-making, a risk assessment model should satisfy several criteria. Since risk is intrinsically a quantitative concept, risks should ideally be expressed quantitatively in numerical units such as expected illnesses and fatalities per capita-year (for individual risks) or per year (for population risks). (Expected values suffice when the random process generating illnesses and deaths is a Poisson process, as the mean number per unit time uniquely determines the entire probability distribution and smaller mean values are always preferred.) Risk estimates should be science-based, i.e., derived from published, publicly available data using explicit objective formulas (e.g., "risk = exposures-per-year x clinical-consequences-per-exposure") to structure and combine data elements. Uncertainties about risks should be characterized by confidence intervals or probability distributions. For rational decision-making, it is important to consider

human health benefits (i.e., risk reductions) as well as risk increases predicted to be caused by proposed risk management actions. Prescriptive risk assessment used to guide risk management decision-making should be based on predicted causal impacts (not just past statistical associations) of proposed changes in animal drug use (not just current situations).

RISK ASSESSMENT MODELING ERRORS TO AVOID

Risk assessment producers and consumers should avoid risk estimates and assessments performed without any clearly stated scope or decisions to be supported; risk models based primarily on assumptions rather than driven by data (e.g., parametric rather than non-parametric models; invalidated statistical models without corrections for model uncertainties, specification errors, selection biases, errors in dependent variables, etc.); attribution of risks to specific causes or sources without empirical evidence or consideration of plausible alternatives; attribution of effects to causes based on statistical and epidemiological associations; causal conclusions made without causal analysis; recommendations based on descriptive data about the current situation, rather than on predictive modeling of the consequences of recommended actions; recommendations for policies and interventions made without decision analysis; and circular citations (e.g., using one published speculation or recommendation to support another without rooting any of them in solid data.)

Recently, many food safety regulators have sought to reduce the amount of work and data required and to streamline risk assessment by making simplifications such as the following in the traditional framework:

- Scoping: Study only one organism and one issue (resistance) at a time and make regulatory decisions (e.g., to ban or restrict animal drugs) based on these single-issue studies. Focus on potential human health risks but ignore potential human health benefits of animal drug use.
- Hazard identification: Identify pathogens that "could potentially be introduced" into food, without showing evidence of a causal relation between exposure to them and increase in human health harm.
- Exposure: Estimate "probability of contamination" but do not quantify the amount of contamination (quantitative microbial load estimates in relation to potential to cause illness).
- Dose-response: Do not use quantitative dose-response information. Instead, substitute judgments that attribute some fraction of total illnesses to contaminated foods and/or bacteria of interest.
- Consequence modeling: Do not quantify the clinical consequences of specific food borne pathogens, specific drug resistances, etc. Instead, substitute holistic judgments (e.g., about the general importance of an entire class of compounds in human medicine) and treat them as surrogates for the specific information of interest (i.e., the change in clinical human health consequences, if any, caused by the specific food borne bacteria and resistance issues that regulation is intended to address).
- Risk characterization: Do not quantify human health risk (frequency and severity of impacts caused by exposures.) Substitute vague holistic and/or judgmental terms such as "high" or "unacceptable".
- Uncertainty analysis: Ignore model uncertainties. Assume that a simple model, e.g., Health damage = k*(contaminated food consumed), is correct.

Such simplifications can certainly lead to easier assessments. But, the results generally cannot improve regulatory decisions, which become matters of subjective judgment rather than science. They can mislead by producing risk estimates that are insensitive to relevant facts and data. For example, focusing on "probability of contamination" rather than probability of harm can make virginiamycin seem potentially very dangerous to immunocompromised humans. Yet, quantitative risk assessment shows that a ban would save fewer than 0.3 statistical lives in the entire US over 5 years (4). Such quantitative information provide risk managers with a different, potentially more useful, perspective on the value of banning animal drugs than assessments based on considerations other than frequency and severity of human health harm. Such quantitative risk information is essential for guiding rational regulation.

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IMPACTS OF ANTIMICROBIAL GROWTH PROMOTER TERMINATION IN DENMARK

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In November 2002, the World Health Organization (WHO) convened an independent, multidisciplinary, international expert panel to review the potential consequences to human health, animal health and welfare, environmental impact, animal production, and national economy resulting from Denmark's program for termination of the use of antimicrobial growth promoters in food animal production, particularly swine and broiler chicken.

Through voluntary and regulatory action, antimicrobial growth promoters were withdrawn from use in cattle, broilers and finisher pigs in February 1998. Use in weaner pigs ceased in the following year. Virtually no antimicrobial growth promoters have been used in Denmark since the end of 1999.

Most information for the review was provided at the International Invitational Symposium; Beyond Antimicrobial Growth Promoters in Food Animal Production, held November 2002 in Foulum, Denmark. This was supplemented where necessary by additional published and (rarely) unpublished data.

IMPACT OF ANTIMICROBIAL GROWTH PROMOTER TERMINATION ON USAGE OF ANTIMICROBIALS

Overall, antimicrobial use in food animals in Denmark has been reduced substantially following the discontinuation of antimicrobial growth promoters. This has resulted in both reductions in the total amount of antimicrobials used and in the average duration of exposure of animals to antimicrobials. On a national basis, the quantity of antimicrobials used in food animals in Denmark has declined 54% from the peak in 1994, (205,686 kg) to 2001 (94,200 kg). Prior to antimicrobial growth promoter termination, most pigs and broilers were exposed to antimicrobials for most of their lives, while after termination the average use of

antimicrobials declined to 0.4 days in broilers (life span usually about 42 days to 2kg), and 7.9 days in pigs (life span usually about 170 days to 100kg).

Termination of antimicrobial growth promoters in pigs resulted in increases in therapeutic use of some antimicrobials that are also used in humans (e.g. tetracycline, penicillins, macrolides), however use of other drugs of importance to humans (e.g. cephalosporins, fluoroquinolones) was unaffected, and total therapeutic use in 2000 and 2001 was similar to 1994, the peak year of therapeutic use before any antimicrobial growth promoters were terminated. Therapeutic use in poultry appeared to be unaffected by antimicrobial growth promoter termination.

IMPACT OF THE TERMINATION OF ANTIMICROBIAL GROWTH PROMOTERS ON ANTIMICROBIAL RESISTANCE

Extensive data were available that showed that the termination of antimicrobial growth promoters in Denmark has dramatically reduced the food animal reservoir of enterococci resistant to these growth promoters, and therefore reduced a reservoir of genetic determinants (resistance genes) that encode antimicrobial resistance to several clinically important antimicrobial agents in humans. Although clinical problems in humans related to resistance to antimicrobial growth promoters were rare in Denmark before and after termination, the principal public health goal of antimicrobial growth promoter termination was to reduce resistance in the food animal reservoir in order to prevent such problems from emerging.

Data from healthy humans however are relatively sparse on which to assess the effect of the termination of antimicrobial growth promoters on the carriage of antimicrobial resistant bacteria. There is some indication that termination of antimicrobial growth promoters in Denmark may be associated with a decline in the prevalence of streptogramin resistance among *Enterococcus faecium* from humans. There is also an indication that the termination may be associated with an increase in resistance among *Enterococcus faecalis* to erythromycin (a macrolide), which may reflect in increase in the therapeutic use in pigs of tylosin (another macrolide). However, it should be noted that erythromycin is not a very important antimicrobial for the treatment of enterococcal infections in humans; preferred drugs include ampicillin, amoxicillin, vancomycin, streptogramins (for *E. faecium*), and linezolid. Further larger studies are needed to determine how much of an effect the discontinued use of antimicrobial growth promoters in Denmark will have on the carriage of antimicrobial resistance in the intestinal tract of humans in the community.

The antimicrobial growth promoters that were used in Denmark were active mainly against Gram-positive bacteria (with the exception of the quinoxalines). Therefore, direct effects of the termination of growth promoters on resistance in Gram-negative bacteria (e.g. *Escherichia. coli*, *Salmonella*) were neither expected nor observed. It is probable, however, that termination of antimicrobial growth promoters had an indirect effect on resistance to tetracycline resistance among *Salmonella typhimurium* because of an increase in therapeutic tetracycline use in food animals. The clinical consequence of increased tetracycline resistance is, however likely to be minimal for the therapy of *Salmonella* infections. This is because patients with gastroenteritis are unlikely to be treated empirically with tetracycline (and tetracycline is no longer used to treat persons with diagnosed *Salmonella* infections in Denmark). Increased tetracycline resistance among *Salmonella* is therefore not likely to result in ineffective treatment of *Salmonella* infections. Increased tetracycline resistance among *Salmonella* may result in additional human *Salmonella* infections, however, since persons who take tetracycline for other reasons are at increased risk of becoming infected with tetracycline-resistant *Salmonella*.

IMPACT OF THE TERMINATION OF ANTIMICROBIAL GROWTH PROMOTERS ON HUMAN HEALTH (OTHER THAN RESISTANCE)

Overall, termination of antimicrobial growth promoters appears not to have affected the incidence of antimicrobial residues in foods or the incidence of human *Salmonella*, *Campylobacter*, or *Yersinia* infections in humans. These are the major zoonoses in Denmark that may be associated with consumption of pork and poultry. In an industry aggressively pursuing successful *Salmonella* reduction strategies, antimicrobial growth promoter termination appears not to have affected the prevalence of *Salmonella* in pig herds, pork, broiler flocks and poultry meat, or the prevalence of *Campylobacter* in poultry meat.

IMPACT OF THE TERMINATION OF ANTIMICROBIAL GROWTH PROMOTERS ON ANIMAL HEALTH (MORBIDITY) AND WELFARE

In swine, there was a significant increase in antimicrobial treatments for diarrhea in the post-weaning period after the termination of antimicrobial growth promoters. A less pronounced and transient increase in antimicrobial treatment for diarrhea was also observed in finishers. In broilers, necrotic enteritis

was at most a minor broiler health problem following the termination of antimicrobial growth promoters, largely because producers continued to use ionophores for the prophylaxis of necrotic enteritis and coccidiosis.

IMPACT OF THE TERMINATION OF ANTIMICROBIAL GROWTH PROMOTERS ON THE ENVIRONMENT

There was no evidence of any adverse environmental effects due to the termination of antimicrobial growth promoters, although there is very little data available with which to make an assessment. The effects of antimicrobial growth promoter termination on total nitrogen and phosphorus output in animal manure appear to be negligible. Available national data indicate that surpluses of these nutrients from agriculture continued to decline following termination.

IMPACT OF THE TERMINATION OF ANTIMICROBIAL GROWTH PROMOTERS ON ANIMAL PRODUCTION

The termination of antimicrobial growth promoters resulted in some loss of productivity, primarily in weaners. There has been no major effect of the antimicrobial growth promoter termination on productivity or feed efficiency in finishers. The economic effects of the antimicrobial growth promoter termination on the pig producer would have been variable and presumably may have included some or all of the following: costs associated with modifications of the production systems to increase pig health, decreased feed efficiency, reduced growth rate and increased mortality in weaners, increased use of therapeutic antimicrobials and costs associated with purchasing alternatives to antimicrobial growth promoters. Some of these costs (e.g. increased therapeutic antimicrobials, reduced growth rate) have been measured and were not large, but others, especially some costs associated with modifications of the production systems, are difficult to measure and were not included in this report, although they may have been substantial for some producers. These costs would have been at least partially offset with savings associated with not purchasing antimicrobial growth promoters. Overall, total volume of pork production in Denmark continued to increase in the period following the termination of antimicrobial growth promoters.

Based on available data, the effects of antimicrobial growth promoter termination on poultry production appear to be small and limited to decreased feed efficiency (-2.3%) that is offset, in part, by savings in the cost of antimicrobial growth promoters. There were no changes in weight gain or mortality in broilers that appeared to be related to the termination of antimicrobial growth promoters.

ECONOMIC IMPACTS OF THE TERMINATION OF ANTIMICROBIAL GROWTH PROMOTERS IN DENMARK

The net costs associated with productivity losses incurred by removing antimicrobial growth promoters from pig and poultry production were estimated at 7.75 DKK (1.04 €) per pig produced and no net cost for poultry. This translates into an increase in pig production costs of just over 1%. Some of these costs (e.g. increased therapeutic antimicrobials, reduced growth rate) have been measured and were not large, but others, especially some costs associated with modifications of the production systems, are difficult to measure and were not included in this report, although they may have been substantial for some producers. Results from using a general equilibrium model of the Danish economy suggest that, as a result of this change in costs, pig production would be around 1.4% per annum lower than might be expected and poultry production 0.4% per annum higher due to termination of antimicrobial growth promoters. The latter result is because poultry production is a competitor to pig production both for inputs and consumption and so indirectly benefits from lower pig production. The overall estimated impact for the Danish economy of antimicrobial growth promoter termination is a reduction of 0.03% (363 million DKK (48 million €) by 2010 at 1995 prices) in real Gross Domestic Product.

Any additional cost to production and the national economy may be, at least partially, offset by the benefits of increased consumer confidence in, and demand for, Danish pig and poultry meat produced without antimicrobial growth promoters. Also to be set against the cost are the likely human health benefits to society of antimicrobial growth promoter termination.

APPLICABILITY TO OTHER COUNTRIES

The consequences of antimicrobial growth promoter termination in other countries should be broadly similar to Denmark, but may vary in some respects depending on the health status of animals and prevailing animal husbandry conditions. In addition, the effects of termination on disease and productivity may vary depending on the type of antimicrobials (e.g. pharmacological properties, spectrum of activity against bacteria) that are currently used in a country. The economic effects will depend upon several factors including the effects on performance levels, the cost of any technologies adopted to compensate for the termination of antimicrobial growth promoters, and

these costs may be offset by the benefits of increased consumer confidence and public health.

CONCLUSION

Internationally, there has been considerable speculation about the effects of antimicrobial growth promoter termination on efficiency of food animal production, animal health, food safety and consumer prices. These issues have been addressed in the "Danish experiment", and there have been no serious negative effects. We conclude that under conditions similar to those found in Denmark, the use of antimicrobials for the sole purpose of growth promotion can be discontinued. Denmark's program to discontinue use of antimicrobial growth promoters has been very beneficial in reducing the total quantity of antimicrobials administered to food animals. This reduction corresponds to a substantial decrease in the overall proportion of individual animals given antimicrobials, and in the duration of exposure among animals given antimicrobials. This represents a general change in Denmark from continuous use of antimicrobials for growth promotion to exclusive use of targeted treatment of specific animals for therapy under veterinary prescription. The program has also been very beneficial in reducing antimicrobial resistance in important food animal reservoirs. This reduces the threat of resistance to public health. From a precautionary point of view, Denmark's program of antimicrobial growth promoter termination appears to have achieved its desired public health goal.

The phasing out of antimicrobial growth promoters was done without major consequences. Under Danish conditions, the negative impacts of antimicrobial growth promoter termination are largely attributable to their disease prophylaxis (i.e. disease prevention) properties, with no effect on growth in broilers and only a small effect on growth in pigs. In pigs, where most antimicrobials were used in Denmark, antimicrobial growth promoter termination was associated with a reduction in growth rate and an increase in mortality and diarrhea in weaners, but these changes were not detectable in finishers. Many of these effects were probably due to termination of olaquindox and carbadox. Even if the pig industry had not decided to voluntarily cease antimicrobial growth promoter use in 1998/99, olaquindox and carbadox would still have been withdrawn in 1999 by EU regulation over concerns about potential toxicity to humans from occupational exposure. The other antimicrobial growth promoters have little or no activity against the gram-negative bacterial infections believed to be most important in post-weaning diarrhea of pigs (tylosin may have activity against *Lawsonia*, but it was banned as an antimicrobial growth promoter by the EU in 1999). Therefore, even if there had been no voluntary discontinuation of antimicrobial growth promoter use, other solutions to the problem of increased post-weaning diarrhea would have been needed. In finisher pigs, antimicrobials did not appear to have these disease prophylaxis benefits and discontinued antimicrobial growth promoter use was not associated with a sustained increase in morbidity or mortality. In broilers, antimicrobial growth promoter termination was not associated with increases in morbidity and mortality, however, ionophores (a drug class not used in humans) were used routinely in feed to prevent the parasitic disease coccidiosis, and this probably also provided some protection against the bacterial disease necrotic enteritis. Savings in antimicrobial growth promoter costs largely offset losses in feed efficiency in broilers.

Based on DANMAP data, 107,000,000,000 mg of active antimicrobial growth promoters were used to produce 2,000,000,000 kg of meat (pork and broiler meat) in 1997. Assuming a feed/gain ratio of 2.4 for the wean-to-slaughter period, it required an estimated 4,800,000,000 kg of feed for broilers and for pigs from weaning to slaughter to produce this meat. Therefore, on average, feedstuffs contained approximately 22 mg of active antimicrobial growth promoter in 1997. Concentrations of drug in feed varied somewhat by drug, species and class of animals, however, the greatest volume of animal feedstuffs was used in finisher pigs and 20 mg/kg tylosin (maximum) was the principal antimicrobial growth promoter used in finishers in 1997 (see figure zz).

The impact of antimicrobial growth promoter termination on mortality in pigs and broilers is addressed under "Impact of the termination of antimicrobial growth promoters on animal production (swine and poultry)" because only crude data (i.e. not cause-specific mortality data) were collected for the purposes of monitoring animal production. This section focuses on issues related to morbidity. The animal welfare issues addressed are confined to animal health issues (physical welfare).

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(Note: this reference is a revised version of the paper presented at the conference in Foulum. In response to queries from the Panel, some of the estimated

production costs were updated, the economic analyses were recalculated and some of the text was revised.)

THE IMPACT OF GROWTH - PROMOTING ANTIBIOTICS ON POULTRY BACTERIAL COMMUNITIES

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ABSTRACT

The focus of the antibiotic resistance debate has now shifted from therapeutic to growth-promoting antibiotics. At the heart of the controversy in U.S. is whether the streptogramin, virginiamycin in poultry will ultimately lead to development of resistance to the human analog, synercid, in vancomycin-resistant enterococci that currently plague US hospitals.

We followed three broiler farms located in Georgia during five consecutive grow outs. Two houses on each farm received a growth promoting antibiotic normally used in the poultry industry, and two designated control houses did not receive growth promoting antibiotics during the five grow outs. We investigated the impact that growth-promoting antibiotics had on the microbiota of poultry. We examined major and minor changes in bacterial populations using a molecular approach, 16S rDNA T-RFLP analysis of microbial community DNA isolated from litter and chicken carcasses from commercial flocks raised on feed supplemented with either virginiamycin or another unrelated, growth promoting antibiotic.

Comparison of T-RFLP profiles did not show apparent differences in the structure of the bacterial community between treated and non treated houses during the analyzed grow outs. We conducted a survey for the presence of streptogramin resistance genes in litter and carcass rinses in treated and non-treated houses. There was no apparent correlation between usage and presence of these antibiotic resistance genes in the poultry environment or on the chicken carcass. For example, in litter samples from two farms, the genes *vatB*, streptogramin A resistance gene; *erm*A macrolide, lincosamide and streptogramin B (MLS) resistance gene; and *ermB*, MLS resistance gene; were detected in samples from streptogramins-treated and non-treated houses. We detected the MLS gene *ermB* in chicken carcass rinses from these same houses regardless of antibiotic usage.

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

THE DEBATE ABOUT ANTIBIOTIC USE IN FOOD ANIMALS: THE SCIENCE VS. THE POLITICS

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In 1997 the European Union (EU) banned against the advice of its own Scientific Committee for Animal Nutrition (SCAN) the use of avoparcin in food-producing animals, in 1999 the E.U. banned again against the advice of its own SCAN the use in food-producing animals of the following antibiotics; bacitracin, tylosin, spiramycin and virginiamycin. Since the so called “growth promoter” antibiotic feed additives are known to have disease preventing and health promoting effects, the bans have resulted in increased incidence of enteric diseases, performance and mortality losses in food-producing animals, and a significant increase in antibiotic usage for therapy. The bans have not resulted in a measurable improvement in human health or a decrease in antibiotic resistance in people affected by infectious diseases. The only measurable benefits from the bans have been a reduction of antibiotic resistance in enterococci from raw meat and intestinal carriers.

It seems like almost every week we read or hear a news report about the dangers of using antibiotics in food-producing animals. The stated concern is that antibiotic use in food-producing animals could create antibiotic resistance in the bacteria present in those animals and that those bacteria could end up in the people that eat their meat. Subsequently, when the people need treatment with an antibiotic, the treatment may fail because the bacteria were resistant.

Theory vs. reality. Although the theory outlined above seems reasonable at first glance, when one starts examining the facts closer a different picture emerges.

Of the most serious 20 bacterial infections exhibiting problems with antibiotic resistance in human medicine, 12 are in no possible way related to antibiotic use in food-producing animals as these bacteria cannot be acquired via the food chain. Of the remaining 8, assuming that transfer of bacterial resistance from animals to people occurs (an unproven assumption in most cases), the calculated percent contribution to antibiotic resistance in all cases is 1% or less, in most cases less than 0.5% (4). Likewise, results from the SENTRY Antimicrobial Surveillance Program, which since 1997 has analyzed worldwide data on antibiotic resistance patterns from both, human and animal bacterial isolates has found little significant association between human and animal patterns (13). According to Ron Jones, MD, results from the SENTRY Antimicrobial Surveillance Program "clearly show a disconnect between antibiotic resistance patterns in humans and animals, calling into question the alleged link between resistant bacteria in animals and those in humans."

Science vs. politics. The European Union (EU) first banned the use of avoparcin (a widely used antibiotic feed additive) against the advice of its own Scientific Committee for Animal Nutrition (SCAN). Invoking the precautionary principle, the EU banned the use of avoparcin in animal feeds in 1997. The rationale behind the ban was that avoparcin use in food-producing animals had created a large reservoir of resistance in the animal and human populations for vancomycin, another antibiotic in the same class, used in human medicine to treat potentially life-threatening nosocomial infections caused by *Enterococcus faecalis* and *Enterococcus faecium*. Avoparcin was banned despite the opinion of EU experts who found no scientific evidence to support the ban. It is interesting to note that despite the fact that avoparcin has never been used as an antibiotic feed additive in food-producing animals in the United States, Vancomycin-Resistant Enterococcal (VRE) infections are far more common and problematic in the US than in the EU.

The 1999 EU ban on virginiamycin reveals a very similar story. A detailed review of scientific evidence by the SCAN concluded that there was no new scientific evidence of virginiamycin transfer from animals to humans (http://europa.eu.int/comm./food/fs/sc/scan/out14_en.html). In addition, the SCAN pointed out that in the US and France where virginiamycin has been used in food-producing animals for over 30 years, the efficacy of antibiotics used in human medicine belonging to the same class had not been compromised. In fact, the results of a very extensive US and Canadian survey on streptogramin resistance in *E. faecium* (12) showed that after 30 years of continuous virginiamycin use in food animals, only 0.2% of over 1000 human clinical isolates tested were resistant to Quinopristin/Dalfopristin (Q/D), the newest streptogramin antibiotic introduced in human medicine to treat VRE infections.

In addition to virginiamycin, the EU also banned in 1999 the use of 3 other antibiotic feed additives used in food animals; bacitracin, spiramycin and tylosin. The remaining antibiotic feed additives are scheduled for withdrawal in 2006, including several ionophore antibiotics.

All of the above has occurred in spite of detailed scientific reviews showing the lack of conclusive scientific evidence of antibiotic resistance transfer from animals to humans. One of these reviews was conducted by the Heidelberg Appeal Nederland (3) that groups almost 200 academics and concluded that "documented *in-vivo* cases showing spread of antimicrobial resistant Gram-positive bacteria from livestock to humans are in essence non-existent." Likewise a comprehensive literature review by the National Research Council in the United States (14) concluded that "the use of drugs in the food animal production industry is not without some problems and concerns, but it does not appear to constitute and immediate public health concern." Another review by the US General Accounting Office (11) stated in some of its conclusions that "Debate exists over whether the role of agricultural use in the overall burden of antibiotic-resistant infections of humans warrants further regulation or restriction," and that "In developing a federal response, both human health concerns and the impact on the agriculture industry are factors to consider."

Consequences of bans on animal health and antibiotic use. The most obvious and immediate consequences of the EU bans on antibiotic feed additives for food-producing animals were reflected in animal health and therapeutic antibiotic use. The bans have led to an increased incidence of enteric diseases in food-producing animals. In addition to productivity and mortality losses, the bans have resulted in an increased use of antibiotics for therapeutic purposes.

In Sweden, Wierup (17) reported increased incidence of necrotic enteritis in broiler chickens requiring antibiotic treatment. Likewise, Inborr (4) reported increased incidence of diarrhea in piglets. In Denmark, although overall antibiotic use is lower than it was before the bans, therapeutic antibiotic use has increased (10). In addition, a 10% increase in diagnosis of ileitis in young pigs has been reported (DS Laboratory – Kjellerup). As in other EU countries, the French broiler industry experienced a 51% increase in use of antibiotics for treatment of necrotic enteritis after the bans. In Holland, consistent increases in tons

of antibiotics used for disease treatment were seen, despite the fact that animal numbers had decreased (National Statistics of Animal Health Products, 2001). Germany saw a 13% increase in use of therapeutic antibiotics in food animals following the bans (I & G Report, 2001). In the United Kingdom, according to the records kept by the Veterinary Medicines Directorate following the 1999 bans, there was nearly a 10% increase in use of therapeutic antibiotics from 1999 to 2000 with food animals accounting for 94% of sales (http://www.vmd.gov.uk/sarss.htm). An increase of 54 tons of antibiotics for therapeutic usage overshadowed a 4-ton decrease in antibiotic usage for growth promotion. Finally, in Switzerland pork producers used 6.1 tons more of antibiotics for therapeutic purposes completely negating the 6-ton reduction of feed additive antibiotic use that resulted from the ban (Animal Pharm, May 10, 2002).

All of the above is in agreement with the findings reported in a recent scientific manuscript by M. Casewell *et al.* (5) showing that the bans on feed additive antibiotics for food-producing animals have had adverse repercussions on animal health. According to the authors, there has been an increase in morbidity and mortality in swine, primarily associated with enteric infections, as well as an increased incidence of necrotic enteritis outbreaks in poultry. The increased incidence of enteric infections has resulted in a significant increase in the volume of antibiotics used for therapeutic purposes. For example, Denmark has seen a 95.8% increase in antibiotic usage for treatment of enteric diseases. Unfortunately, the increased has come primarily from those classes of antibiotics more frequently used in human medicine (tetracyclines, macrolides, lincosamides and aminoglycosides). Therefore, instead of reducing the risk of antibiotic resistance for humans, the bans appear to have had the opposite effect as reported by the same authors in regards to the increased incidence of antibiotic resistance in *Salmonella* and *Campylobacter*.

Consequences of the bans on human health. Supposedly all the bans on antibiotic feed additives for food-producing animals were implemented for the sole purpose of reducing antibiotic resistance problems in human medicine. Therefore, 4 years after the bans were implemented, it is reasonable to ask: Has the objective been achieved? Not according to a recent scientific manuscript by Casewell, *et al.* (5) who reported that there has been "no diminution in the prevalence of resistant enterococcal infection in humans;" instead, according to the authors "vancomycin resistance appears to be increasing in enterococcal infections in parts of Europe over the period of the ban."

What about the incidence of, and antibiotic susceptibility of the major food borne illnesses, salmonellosis and campylobacteriosis? According to the same authors, "human salmonellosis has not responded to control measures in some parts of Europe, and microbiologically confirmed infections actually increased in prevalence in Denmark in 2001 after they had declined for 3 years." The same authors state that regarding *Campylobacter* the situation may be even worse "in Denmark, it has steadily increased in prevalence over the past decade and there is more tetracycline and fluoroquinolone resistance in human than in animal isolates."

The European Union's double standard. A double standard exists in regard to antibiotic use in food-producing animals in the EU, as poultry, swine and cattle are in most cases not raised "antibiotic-free" but rather raised without antibiotic growth promoters. As shown earlier, the lack of antibiotic feed additive use has resulted in higher incidence of enteric disease outbreaks in food-producing animals; this in turn has resulted in the use of higher levels of antibiotics, and the use of antibiotics of much more importance in human medicine than the ones used in the feed before the bans. As pointed out by Casewell *et al.* (5), "The published evidence suggests that the growth-promoter bans have reduced overall antibiotic use in food-producing animals. It is increasingly clear, however, that the use of growth promoters was accompanied by other, previously unrecognized, health promotional or prophylactic effects."

The focus on food-producing animals. From the results previously discussed, it is clear that even if one assumes that antibiotic resistance transfer from animals to people occurs (an unproven assumption in most cases), the potential contribution of food-producing animals to the overall antibiotic resistance problem would be minimal to nil. On the other hand, a 2-year long survey on antibiotic resistance in a community, conducted by researchers from Wales and published in the British Medical Journal (16) clearly documents the positive correlation between antibiotic prescribing practices in a community and the development of antibiotic resistance in the same community. The number of prescriptions written on a yearly basis per 1000 patients produced practically a mirror image when compared to the average resistance rate in bacteria isolated from surgical samples from the community hospital. In all cases, the higher the number of prescriptions written for a given antibiotic, the higher the average resistance rate in the bacteria tested from the surgical samples.

Therefore, it is distressing to see that for the most part the antibiotic resistance debate has been restricted to antibiotic use in food-producing animals. Clearly, and even with the acknowledgement of the World Health Organization (WHO), antibiotic prescription by medical doctors in human practice is the driving force

behind the antibiotic resistance problem. Also clearly, of the two distinct animal populations, food-producing animals and companion animals, and as pointed out by others (1,2,6,15), companion animals are a much more likely source of antibiotic resistance transfer to humans than food-producing animals.

First of all, recent polls among the American people have shown that a large percentage of the population consider their pets as members of the family, and a significant percentage has admitted to letting their pets sleep in bed with them or in their bedrooms. Many pets live in intimate contact with their owners. In many cases, there is frequent tongue to face and mouth-to-mouth contact. In addition, indoor litter boxes for pets are a common sight in many households. Many pets live indoors and from time to time pets defecate and urinate indoors. Ask yourself, where is the chance for a significant germ exchange greater, between a baby or a child and his/her puppy or kitten? Or between that baby or child and a raw piece of chicken? I have witnessed many times dogs licking their owners' faces and lips, including babies and children, but I am yet to witness a baby or a child lick a piece of raw chicken. All of us should remember that before chicken is consumed, it is cooked, and during this process the bacteria that might have been on it are destroyed.

Dogs, cats, and other companion animals get treated with the same classes of antibiotics often prescribed in human medicine with little to no supervision by any regulatory agency, in much the same way as those prescribed by medical doctors to their patients. So it is difficult to comprehend why scientists and politicians are most concerned with antibiotic use in food-producing animals, instead of antibiotic prescription practices by medical doctors and companion animal veterinarians.

Politically motivated bans vs. scientific risk assessments. It seems like the activists groups determined to make animal agriculture an unprofitable business, and frustrated by their lack of success on the scientific front with the US Food & Drug Administration Center for Veterinary Medicine (FDA/CVM), have now turn their efforts to lobbying Washington politicians to impose their views on the rest of us by legislative mandate.

Currently under review in the House of Representatives in Washington, DC, is a bill (H.R. 2932) sponsored by Rep. Sherwood Brown (D-OH) entitled "The Preservation of Antibiotics for Medical Treatment Act" that proposes to eliminate use of eight feed additive antibiotics considered "growth promoters" with equivalent counterparts in human medicine. The same bill is being sponsored in the senate by Sen. Ted Kennedy (D-MA). It is a clear attempt by activist groups opposed to animal agriculture, to bypass the regulatory system that has established the FDA/CVM as the federal agency responsible for dealing with veterinary drug approvals and withdrawals, and the United States Department of Agriculture Food Safety & Inspection Service (USDA-FSIS) as the federal agency responsible for ensuring the safety of the food supply in the US.

FDA/CVM has emphasized the need for scientific risk assessments prior to determining the fate of any animal drug already approved for use in food-producing animals. Comprehensive risk assessments have been completed for two of the antibiotics used in food-producing animals of most concern to the scientists at the FDA/CVM and the Centers for Disease Control & Prevention (CDC).

We believe that although misled politicians may be willing to sponsor legislation like HR 2932 for political gain, the transparency of the scientific process must remain unchanged and in the hands of the capable scientists at the FDA/CVM. The first scientific risk assessment, for the fluoroquinolone antibiotic enrofloxacin (Baytril®) showed that the human health risk from using this antibiotic in food-producing animals is practically nil. The second one, for the streptogramin antibiotic virginiamycin (Stafac®) was also completed and reported (7,8,9). Even when worst-case scenario conditions were used, like assuming that resistance transfer from animals to people occurs (an unproven assumption in this case), the risk assessment proved scientifically and conclusively that the human health risk from virginiamycin use in food-producing animals was negligible. Perhaps, the activist groups and the politicians willing to sponsor their views on antibiotic use in food-producing animals should learn from the European experience, that it is clearly beginning to show the adverse consequences of the antibiotic feed additive bans on both, animal health and human health.

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THE RELATIONSHIP BETWEEN POULTRY HEALTH AND FOOD SAFETY

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SUMMARY

As more is learned about the science linking flock health and finished product microbial prevalence and load, the Veterinary Avian Specialist's role in Food Safety will enlarge. Recent research shows a surprising relationship between a number of common poultry diseases and carcass microbial loads in the processing plant. It follows that maintaining optimal flock health is essential to minimizing food borne pathogens. If pre-harvest HACCP is to be meaningful, the live-production critical control points that impact food safety must be recognized. Management methods, disease prevention biologics, antimicrobials, and therapeutics are valuable veterinary tools, and must remain available. Following is a review of what is currently known about the inter-relatedness of flock health and carcass microbial contamination.

REVIEW

A variety of bacterial, viral, and "management" diseases of poultry cause morbidity and mortality, increase feed conversion, and reduce body weights and the uniformity of the flock at the time of harvest and processing. Northcutt states that, "Variation in bird size (uniformity) within a flock or over time can affect the efficiency of processing plant equipment,

specifically at the vent opener during evisceration," and that "Frequency of carcass contamination depends upon the amount of material present in the digestive tract, the condition of the digesta (partially digested food and feces) remaining in the intestines (watery or firm), the integrity of the intestines, and the efficiency of the eviscerating equipment and plant personnel" (1).

How does disease impact these conditions? As in other species, bacterial and viral infections in poultry produce a fever, leading to cachexia ("off feed") in a portion of the flock. Northcutt and Bilgili find that "When the length of feed withdrawal is too long (greater than 13 or 14 hours), a number of problems may occur that increase the likelihood of carcass contamination. . . .Weaker intestines have a higher incidence of intestinal tearing during evisceration. . . . Intestinal strength of broilers has been found to be approximately 10% lower when broilers were without feed for 14 or more hours before processing as compared to full-fed broilers (1, 2, 8, 9)." So any disease that takes birds "off feed" can potentially impact carcass contamination prevalence in the plant.

Carcass pathogen load can be affected as well. Byrd *et al*. reported that *Campylobacter* positive crops increase from 25% before feed withdrawal to 62.4% after feed withdrawal (3). Corrier *et al*. found that *Salmonella* positive crops increased from 1.9% to 10% following feed withdrawal (4). Stern *et al*. produced a five-fold increase in *Campylobacter* positive carcasses of cooped broilers off-feed for 16-18 hours, versus broilers on litter with full feed (5). Humphrey *et al*. (6) and Hinton *et al*. (7) found elevated *Salmonella* levels in broiler crops during feed withdrawal, perhaps due to the higher crop pH in feed-withdrawn birds causing a microflora shift. In commercial layers, the molting process involves dramatic reductions in feed consumption, and results in amplification of *Salmonella enteriditis* (SE) infections and horizontal transmission (10, 11) and increases the onset and degree of intestinal inflammation from SE infection (12). Coccidiosis (*Eimeria tenella* challenge) in chickens also infected with SE resulted in higher cecal SE populations (13), recrudescence of previous SE infections (14), and increased invasiveness of *Salmonella typhimurium* (ST) into the cecal wall (15). Coccidiosis control should be considered a pre-harvest critical control point.

Bilgili states that, "Preventing fecal contamination of the carcasses from spillage of digestive tract contents or smearing of fecal material on edible meat surfaces is the single most important aspect of sanitary slaughter and dressing regulations (16)." In *Generic HACCP Application in Broiler Slaughter and Processing* (National Advisory Committee on Microbiological Criteria for Foods), McNamara states, "Evisceration can be a major source of additional fecal contamination, particularly if the intestines are cut. This would be expected to increase contamination by mesophilic bacteria, including intestinal pathogens (i.e., *Salmonella, Campylobacter,* and *C. perfringens)*. Cut intestines can lead to contamination of equipment, workers, and inspectors and can be a major source of cross-contamination (17)." A variety of disease conditions produce non-uniform broiler flocks (size and weight). These birds present poorly to mechanized plant equipment that is set for the "average" bird, and as the distribution curve for uniformity flattens, the equipment cuts and tears more intestinal tracts. To test this observation, Russell conducted a replicated processing plant study, *The Effect of Airsacculitis on Bird Weights, Uniformity, Fecal Contamination, Processing Errors, and Populations of Campylobacter and Escherichia coli* (18).

Russell summarizes his findings: "The net loss (airsac positive carcass weights) averaged over five repetitions was 84 g/carcass, equating to a loss of 14,686.9 k (32,379 lb) of chicken meat for one growout house per year as the result of AS (airsac) infection. ASP (airsac positive) carcasses had higher (p< or = 0.05) fecal contamination in four of five repetitions. The number of total digestive tract cuts or tears were much higher on ASP carcasses at 42, 49, 37, 60, and 59% as compared to 14, 12, 17, 24, and 16% for ASN (airsac negative) carcasses in repetitions 1 to 5, respectively. In three of the five replications, the presence of AS in the flocks increased (P< or = 0.05) the number of *Campylobacter* recovered from broiler carcasses. Hence, there appears to be a relationship between the presence of AS and *Campylobacter*-positive carcasses. . . .Because flocks of chickens showing signs of AS have lower weights, more fecal contamination, more processing errors, and higher levels of *Campylobacter* spp., broiler companies should emphasize control of AS in the flocks as a means of preventing subsequent food-borne bacterial infection (18). Pilot studies conducted by Russell in two integrated broiler companies in 1997 demonstrated that on carcasses removed by FSIS inspectors for airsacculitis, a total of 96% had questionable or unacceptable *E. coli* counts pre-salvage, versus 42% for inspector-passed, pre-chill carcasses, and that, "In a second study conducted by another integrator, pre-chill *E. coli* counts for carcasses with airsacculitis were significantly higher (P< or = 0.05) at 3.93 log 10 CFU/mL than airsacculitis negative carcasses at 2.63 log10 CFU/mL. Moreover, this company found that *Salmonella* prevalence for carcasses with airsacculitis was significantly higher (P< 0.05) at 70% than for carcasses without airsacculitis at 40% (unpublished data). These studies demonstrate a link between the presence of airsacculitis in the flock and increases in indicator and pathogenic bacterial populations (19). In

another processing plant study, Russell used FSIS condemnation records and whole-bird rinse *Salmonella* counts collected over two years on 32 million birds processed (19). He applied statistical analysis to determine the statistical relatedness of six parameters: airsacculitis, infectious process (I.P.), total condemnation, *Salmonella* prevalence, carcass weight, and fecal contamination. Russell concluded, "The analyses showed that as the percentage carcasses removed from the line by the U.S.D.A. inspectors increased, the percentage of carcasses with fecal contamination increased as well. Increasing levels of infectious process also resulted in a significant increase in fecal contamination. The data revealed that when a high number of carcasses are condemned, an increased fecal contamination occurred. A significant finding was that, as the number of carcasses removed from the line for active airsacculitis increased, the prevalence of *Salmonella* on processed carcasses increased as well. The statistician concluded that 'with samples of the size used in this investigation, these differences are quite significant; there is very convincing evidence that airsacculitis increase is associated with increasing probability of *Salmonella* (contamination)'. . . .From the above studies, it becomes apparent that the reduction of airsacculitis in broiler flocks entering the processing plant is a food safety concern (19).

CONCLUSION

As Hargis summarizes, "Research has clearly demonstrated that the reduction of microbial contamination of processed poultry requires the identification of both pre- and postharvest critical control points where contamination may occur, and the implementation of integrated control programs (20)." Known factors affecting carcass microbial contamination or food pathogen prevalence are airsacculitis, infectious process (IP), coccidiosis, total condemnation, flock uniformity, cut intestinal tracts, fecal contamination, extended feed withdrawal and the molting process. Disease prevention and treatment are therefore essential to meeting Food Safety goals, as are the use of current and future vaccines and drugs.

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IS THERE HUMAN HEALTH HARM FOLLOWING FLUOROQUINOLONE USE IN POULTRY?

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SUMMARY

CVM has not brought forth new evidence to substantiate their concern for the safety of the continued use of fluoroquinolones to treat serious infections in poultry. Rather than raise a question about the safety of fluoroquinolones, new evidence only reaffirms the importance of these products for assuring a safe food supply. New data show that 1) in the US, there is a disconnect between poultry and human disease and poultry is not as important a source for campylobacteriosis as previously believed; 2) when the data are analyzed correctly, there is no human health harm; and 3) when effective therapy for airsacculitis is not applied, the potential for food borne illness increases dramatically.

INTRODUCTION

In 1994, prior to approving any fluoroquinolones for use in food-producing animals, the US Food and Drug Administration's (FDA's) Center for Veterinary Medicine (CVM) assembled a Joint Advisory Committee consisting of members of the Veterinary Medicine Advisory Committee and FDA's human Anti-Infective Drugs Advisory Committee. The Joint Advisory Committee members were charged with making a science based recommendation on whether fluoroquinolones should be approved for use in food-producing animals. Following a two-day public hearing, the Committee concluded that fluoroquinolones could be safely used in food-animal production provided certain restrictions were established. These restrictions included use for therapy only, use under the supervision of a licensed veterinarian, extra-label use should be prohibited and a resistance monitoring system should be established (1). The fluoroquinolone enrofloxacin, trade name Baytril®, was subsequently approved for use in chickens and turkeys in 1996 with the recommended restrictions in place.

In October of 2000, CVM proposed to withdraw the approval of enrofloxacin for use in poultry by issuing a Notice of Opportunity for Hearing (NOOH). CVM believed that new evidence, which became available post-approval, raised concerns that the use of fluoroquinolones in poultry was contributing to fluoroquinolone resistant *Campylobacter* in poultry, that humans were being exposed to fluoroquinolone resistant *Campylobacter* through the consumption of poultry, and that an adverse human health event was occurring when patients with clinical signs of gastroenteritis were treated empirically with fluoroquinolones. The NOOH is a due process offered by the FDA to scientifically explore the concerns brought forth. The FDA has full authority to withdraw any product from the marketplace, without a hearing, if they feel the public is in imminent danger by continued use of the product. The NOOH offered Bayer the opportunity to explore these new concerns put forth by CVM to determine if they were justified. After reviewing all available data and consulting with appropriate outside experts, Bayer concluded that the new information did not substantiate CVM's concerns. To the contrary, the data reaffirmed the safety of enrofloxacin when used in poultry. Consequently, in February 2001 Bayer requested that a hearing be granted based on the compelling scientific evidence. In February of 2002 CVM responded to the request for a hearing by issuing a Notice of Hearing (NOH) which initiated the hearing process.

The team of outside experts that Bayer consulted was comprised of: 1) physicians and microbiologists with extensive experience in studying campylobacteriosis in the USA and Europe; 2) statisticians, risk assessors, and epidemiologists that

extensively analyzed data generated by the Centers for Disease Control and Prevention (CDC) and CVM; 3) numerous poultry experts including veterinarians from production, research institutes and academia; and 4) internal medicine physicians responsible for treating enteric diseases on a daily basis.

CHICKEN AS A SOURCE

As the above experts began to look at the US data, there appeared to be a disconnect between poultry consumption and campylobacteriosis in people. FoodNet data, published by CDC, showed a 26% reduction in overall human campylobacteriosis in the US from 1996-97 to 1999 (a decrease from 2.4 to 1.4 million estimated cases) (2). FoodNet data also showed that the rate of campylobacteriosis per 100,000 dropped from a high of 25.2 in 1997 to 13.4 in 2002 (a 47% reduction) (3). This reduction occurred at the same time that chicken consumption was on the rise. Per capita consumption rose 7.4% between 1996 and 2001 (48.8 pounds [22.2 kg] vs. 52.4 pounds [23.8 kg], respectively) (4). An increase in chicken consumption, while campylobacteriosis is on the decline, raises serious questions, contrary to previous beliefs, about poultry as the primary source of human disease in the US.

Two major *Campylobacter* case-control studies were conducted or funded by CDC. These studies evaluated the risk factors for campylobacteriosis, the risk factors for contracting a fluoroquinolone resistant *Campylobacter* infection, and/or the human health impact of contracting a fluoroquinolone resistant *Campylobacter* infection. In the first study, Friedman *et al*. enrolled 1,316 patients from seven FoodNet surveillance sites (5) while Effler et al studied 211 patients from Hawaii (6). Both studies found that eating chicken prepared in a restaurant or outside the home was a risk factor for campylobacteriosis. Friedman also found that the risks of acquiring campylobacteriosis were similar whether patients eating in restaurants consumed chicken, steak, pork chops, sausage, or pork roast (Odds ratio [OR] of 2.4, 2.2, 2.8, 3.2, and 6.5, respectively). Interestingly, both Friedman and Effler found that eating chicken prepared in the home was "protective" (OR of 0.5 and 0.6, respectively). In other words, where chicken was prepared and consumed in the home, patients were less likely to get campylobacteriosis than where people who did not prepare or consume chicken in the home (5, 6).

Culture results from poultry in retail markets have repeatedly shown that *Campylobacter* are present on poultry meat in a high percentage of the carcasses sampled. Poultry prepared and served in the home is purchased at the retail market but doesn't show up as a significant risk factor for campylobacteriosis. The fact that it doesn't may be explained by a lack of sufficient colony forming units (CFUs) per carcass to cause illness, acquired immunity following low level exposure to *Campylobacter*, and/or the fact that only a small percentage of *Campylobacter* found on poultry are a genetic match to human isolates. United States Department of Agriculture (USDA) data confirm a reduction in both the number of contaminated carcasses and the number of CFUs per carcass, providing further evidence of the diminishing role of poultry as a source of human *Campylobacter* infections (7).

HUMAN HEALTH IMPACT

Critical to the discussion of the use of fluoroquinolones in poultry is the potential for the selection for resistant *Campylobacte*r and the impact these resistant organisms may have on the ability to successfully treat human cases of campylobacteriosis, given that contaminated poultry can be a potential source of human infection.

A major issue concerning data analysis of *Campylobacter* case-control studies is whether or not to adjust for foreign travel when evaluating for human health impact. Friedman suggested in her analysis of the CDC study for identifying campylobacteriosis risk factors: *"Because of their potentially unique exposures and because they were not matched with controls who traveled, the 164 patients who traveled internationally in the 7 days before illness were excluded from further analysis"* (5). Bayer's expert consultants agree with Friedman. Certainly, when evaluating the impact of the domestic use of fluoroquinolones in poultry, campylobacteriosis cases acquired outside the US are not relevant; therefore these cases must be excluded from the analyses.

The findings of a *Campylobacter* case-control study conducted in Minnesota were published by Smith *et al*. in the New England Journal of Medicine in 1999 (8). Smith's data suggest that resistant cases are less likely to be associated with consumption of chicken/turkey compared to susceptible cases (OR 0.2) (9). Smith did not adjust for foreign travel or prior fluoroquinolone use when reporting that patients with a fluoroquinolone resistant infection had a longer duration of diarrhea than those with a fluoroquinolone susceptible infection (median of 10 vs. 7 days, respectively). When adjusting for both foreign travel and prior fluoroquinolone use, the median length of illness was 6.9 days for resistant infections vs. 7.0 days for susceptible infections (9).

CDC examined the FoodNet data generated from their *Campylobacter* case-control study to evaluate the human health impact of having a ciprofloxacin resistant *Campylobacter* infection compared to having a ciprofloxacin susceptible infection. A number of

analyses conducted by CDC on the data set have demonstrated that, even when the data are not adjusted for foreign travel, statistically, there are no differences in duration of diarrhea between patients with susceptible and resistant infections when patients, not taking antidiarrheals, are treated with fluoroquinolones (10). CDC's own analysis of duration of hospitalization, without controlling for foreign travel or prior fluoroquinolone use, also showed that patients with susceptible infections were hospitalized longer than those with resistant infections. [Median of 3 vs 2 days; p value = 0.01 (11).]

HUMAN HEALTH BENEFITS OF FLUOROQUINOLONE USE IN POULTRY

An important aspect that has not been considered at all by CVM is the negative human health impact of removing fluoroquinolones from use in poultry. Fluoroquinolones are often the only effective antimicrobials for the treatment of airsacculitis caused by *E. coli* in broilers. A processing plant study conducted by Russell (12) and analyzed by Cox and Popken (13) have shown that broiler carcasses originating from flocks with airsacculitis, as a result of either no or unsuccessful treatment, are more likely to be contaminated with *Salmonella* and *Campylobacter*. Lack of effective treatment for airsacculitis results in more underweight birds, with weakened intestines, being processed. This along with an increase in processing errors due to the lack of uniformity in bird size leads to greater fecal contamination of meat during processing and thereby presents an increased risk of food borne illness from not just *Campylobacter*, but other pathogens as well. Cox and Popken have shown that the benefits of having enrofloxacin available to successfully treat airsacculitis far outweigh the risks.

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AUSTRALIAN INITIATIVES TO CONTROL ANTIBIOTIC RESISTANCE ISSUES IN THE POULTRY INDUSTRY

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SUMMARY

In response to international pressures on the use of antibiotics, particularly "growth promotant" antibiotics in food-producing animals, Australia undertook a review of antibiotic resistance commencing in 1997 which resulted in a range of recommendations being implemented commencing in 2000. A number of the recommendations that relate to poultry have been fully implemented or are close to being so, which has resulted in additional restrictions on the use of antibiotics in food-producing animals. While the Australian poultry industry can control most of the important bacterial diseases of poultry by vaccination, biosecurity, and hygienic measures, the control of some intestinal infections such as those caused by *Clostridium perfringens* is still highly dependant on the use of the remaining permitted in-feed antibiotics. Means of control of intestinal bacterial infections other than by antibiotics are being monitored, trialled, and researched by Australian chicken meat processors. The Australian Veterinary Poultry Association and the Australian Chicken Meat Federation have co-operated fully with government during this process.

INTRODUCTION

The development and spread of antibiotic-resistant bacteria in humans and the consequential difficulty in controlling some bacterial infections, particularly in hospitals, have become increasingly frequent topics for scientific discussion and media reports in recent years. While it is likely that antibiotic resistance has occurred mainly due to the use, overuse and even improper use of antibiotics in humans and the failure of the medical profession to employ adequate infection-control procedures to prevent the spread of antibiotic-resistant bacteria in hospitals, the use of antibiotics in animals has been incriminated as a possible cause of antibiotic resistance problems in human medicine.

It was in this context that in December 1997 a joint Human Health and Agriculture committee, named the Joint Expert Technical Advisory Committee on Antibiotic Resistance or JETACAR (http://www.health.gov.au/pubs/jetacar.htm), was established in Australia to review the scientific evidence on the link between the use of antibiotics in food-producing animals and the emergence and/or selection of antibiotic-resistant bacteria and their spread to humans. In addition, JETACAR was to develop evidence-based recommendations for the appropriate future management of antibiotic use in food-producing animals.

ANTIBIOTIC USE

Most economically important bacterial diseases of poultry in Australia, such as avian cholera (*Pasteurella multocida*), chronic respiratory disease (*Mycoplasma gallisepticum*), infectious synovitis (*Mycoplasma synoviae*), infectious coryza (*Haemophilus paragallinarum*), duck infectious serositis (*Reimerella anatipestifer*) and erysipelas (*Erysipelothrix rhusiopathiae*) are well controlled by vaccination, biosecurity and hygienic measures, as are to some extent *Salmonella* infections. Therapeutic antibiotic medication is required only when these control measures fail. Australia is fortunate not to have endemic *Salmonella enteritidis* infection of poultry, with localized minor outbreaks having been quickly brought under control, and an apparent absence of the phage types that cause human disease in other countries.

The Australian Pesticides and Veterinary Medicines Authority or APVMA (http://www.apvma.gov.au) has never registered, or has withdrawn from registration, some antibiotics for food-producing animals such as fluoroquinolones, nitrofurans, the glycopeptide avoparcin, gentamicin, and chloramphenicol, while some registered antibiotics such as ceftiofur are not registered in poultry and cannot be used for mass administration.

However, gastrointestinal infections with *Clostridium perfringens* (the cause of necrotic enteritis, cholangiohepatitis and subclinical enteritis) and with less-defined bacteria which cause "dysbacteriosis" (1) have been traditionally prevented in meat chickens largely by the use of in-feed medications to control coccidiosis, caused by the protozoan *Eimeria* spp., and Gram-positive bacterial infections. The use of these antibiotics in meat chickens, which are often registered as "growth promotants," particularly the streptogramin virginiamycin, the polypeptide bacitracin, the macrolide tylosin phosphate, and the orthosomycin

avilamycin has been most contentious in chicken meat production in Australia.

CHICKEN MEAT INDUSTRY

Approximately 450 million meat chickens in Australia are grown per annum under a wide range of climatic conditions in both open and controlled-environment sheds. Wheat is the predominant grain included in rations, but in some locations of Australia sorghum and barley are used seasonally. Sheds are thinned out a number of times from as early as 30 days until about 56 days when bird weight is approximately 3 kg. Chicken meat consumption in Australia is approximately 34kg/person. The population of Australia is 20 million.

ANTIBIOTIC-RESISTANT BACTERIA

Resistant bacteria that have been of major medical concern worldwide include vancomycin *resistant enterococci* (VRE), multiresistant *Staphylococcus aureus* (MRSA), multiresistant *Salmonella typhimurium* DT104, enterohemorrhagic *Escherichia coli* such as O157 and O111 strains that cause hemolytic-uremic syndrome (HUS) and fluoroquinolone-resistant *Campylobacter*. In Australia the predominant genetic subtype of VRE detected in human cases is *vanB,* which is not found in chickens (2) and which is usually sensitive to the glycopeptide teicoplanin. In the only comprehensive survey of contamination of chicken meat with *Enterococcus* spp. in Australia, Barton and Wilkins (2) found a prevalence of 8.5% of processed chickens with any level of vancomycin resistance. *Salmonella typhimurium* DT104 has not been found in chickens (or other livestock) in Australia, despite intensive ongoing *Salmonella* monitoring within the National Enteric Pathogens Surveillance Scheme. *Escherichia coli* types that cause HUS have not been reported in Australian chicken meat. Fluoroquinolone-resistant *Campylobacter* has not been isolated from Australian chicken meat (2,3). There have been no imports of chicken meat into Australia at this stage.

OUTCOMES OF JETACAR

JETACAR did not adopt the EU "precautionary principle" approach, but rather recommended that antibiotic resistance be evaluated by "risk assessment" when antibiotics are being registered or reviewed. This approach and the twenty-two JETACAR recommendations were subsequently accepted in August 2000 by the Australian government and the Commonwealth Interdepartmental JETACAR Implementation Group or CIJIG (http://www.health.gov.au/pubhlth/strateg/jetacar/cijig.htm) established to implement the recommendations.

Progress to date on recommendations that relate to poultry include:

1. The Expert Advisory Group on Antimicrobial Resistance (EAGAR) was established to provide technical input to CIJIG, registration authorities and the National Drugs and Poisons Schedule Committee (http://www.tga.health.gov.au/ndpsc).
2. A Special Review of Virginiamycin by the APVMA is nearing completion. The recommendations are to remove all growth promotion claims and to limit its use pattern to a three weeks "treatment" period at 20mg active/tonne feed for necrotic enteritis control.
3. Tylosin phosphate is currently undergoing a Special Review by the APVMA.
4. Registration authorities have upgraded methods for collecting antibiotic use data. Since all antibiotics are imported into Australia, records on total use of each antibiotic can be compiled, but in addition estimates of use in various animal species are now being collated.
5. The scheduling of all antibiotics used in food-producing animals is being reviewed, based on a risk assessment approach to determine whether they should be available only on veterinary script. Major changes to date include the rescheduling of all inclusion levels of bacitracin and virginiamycin to veterinary script products, while the scheduling of ionophores (polyether antibiotics), avilamycin and flavophospholipol were not changed from non-veterinary script products at common usage levels.
6. "Control of Use" legislation for antibiotics has been harmonized in all Australian states, which includes the application of Label Restraints on some antibiotics, such as virginiamycin and ceftiofur that are used in food-producing animals, to prevent off-label use by veterinarians and producers.
7. A national antibiotic resistance surveillance system has been initiated by government with the co-operation of industry for all major food-producing industries.
8. The Australian Veterinary Poultry Association has updated its Code of Practice for the Use of Antibiotics in the Poultry Industry (http://www.jcu.edu.au/school/bms/avpa) to encompass the JETACAR recommendations and to include Prudent Use principles.
9. Research has been initiated on alternatives to antibiotic growth promotants and for the

control of necrotic enteritis via the Rural Industries Research and Development Corporation (http://www.rirdc.gov.au).

DISCUSSION

The Australian Veterinary Poultry Association, which represents veterinarians servicing the poultry industry, and the Australian Chicken Meat Federation, which represents the chicken meat processors, have co-operated fully with government to review the possibility that antibiotic resistance developed by bacteria infecting meat chickens could pose a food-safety hazard and have assisted in the implementation of the JETACAR recommendations. At this stage, even though a number of antibiotics are not permitted for use or their use has been limited, the Australian poultry industry can control major bacterial diseases by a combination of vaccination, biosecurity, hygienic measures, and medication. However, the chicken meat industry is monitoring the experiences in the poultry industries of a number of EU countries where many in-feed antibiotics have been banned, is aware of some research overseas into alternatives to antibiotics, has trialled alternatives to antibiotics for control of intestinal infections and has initiated research in Australia, particularly on possible non-antibiotic replacements for control of *Clostridium perfringens* infections.

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CLINICAL AND MOLECULAR CHARACTERISTICS OF INFECTIOUS BURSAL DISEASE VIRUSES IDENTIFIED IN LATIN AMERICA

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SUMMARY

Some outbreaks of infectious bursal disease have been observed in Latin America during the last five years. Clinical and pathological findings have been similar to those described in other continents. Using phenolized bursal samples, infectious bursal disease viruses have been identified using reverse transcription-polymerase chain reaction (RT-PCR) followed by restriction fragment length polymorphism (RFLP). The identified viruses varied from classical very virulent (vvIBDV), to Delaware variant strains, depending upon the country of origin. After nucleotide and amino acid sequence analysis, the vvIBDV detected were similar to those described in Europe, Asia and Africa.

INTRODUCTION

Infectious bursal disease (IBD) or Gumboro disease is an acute, highly contagious viral disease of young birds characterized mainly by severe lesions in the bursa of Fabricius followed by immunosuppression (2, 8, 10). Infectious bursal disease virus (IBDV) is a member of the *Birnaviridae* family with a genome consisting of two segments of double stranded RNA (dsRNA). Two serotypes of IBDV have been recognized, but only serotype I causes naturally occurring disease in chickens (5 ,7 ,9).

Because of the high rate of mutations and genetic variability, the different strains of IBDV present in the field display diverse antigenic and pathogenic properties. To establish the proper control procedures, it is important to characterize the antigenic and pathological properties of the strains prevalent in a geographic area; thus, it is necessary to develop rapid and accurate methods for typing the different strains of IBDV. The purpose of this study was to characterize field IBDV isolates from the United States and other countries in Latin America, based upon differences in the hypervariable region of VP2 gene by RFLP analysis and nucleotide and amino acid sequence analysis.

MATERIAL AND METHODS

Bursal tissues from commercial broiler farms suspected of having IBDV in several countries of Latin America were initially inactivated using phenol:chloroform (5:1), pH 4.3 ± 0.2 (Fisher Chemicals, Fair Lawn, N.J.) in order to inactivate the infectivity of the IBDV but preserving the viral genome to be amplified by RT/PCR (6). All the foreign samples were inactivated with phenol-chloroform before they were introduced to the United States.

RNA from phenol:chloroform inactivated bursas was extracted using an acid-guanidium-phenol-chloroform RNA extraction method according to previously published methodology (3). RNA from bursal samples was reverse transcribed to cDNA and amplified by a one step reverse transcriptase-polymerase chain reaction (RT-PCR) (1,4). After amplification, the samples were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

The RT-PCR products were genotyped by restriction fragment length polymorphism (RFLP) using the restriction endonucleases *Dra* I, *Sac* I, *Taq* I, *Sty* I, *Bst* NI, and *Ssp* I (New England Biolabs Inc., Beverly, MA) according to the manufacturer's instructions. Digested fragments were subjected to electrophoresis on polyacrylamide gel and visualized by rapid silver staining. Electrophoretic patterns were compared with reference IBDV strains.

Selected RT-PCR products were purified and then sequenced by the dideoxy-mediated chain-termination method (11). Phylogenetic analysis of the hypervariable of VP2 gene was performed by parsimony method using the package PAUP 4b2 TM (Sinauer Associates Inc. Sunderland, MA).

RESULTS AND DISCUSSION

There was a variety of IBDV genotypes depending upon the country where the samples were obtained. Strains exhibiting genotype characteristic of vvIBDV strains, such as UK-661 strain, were detected in Brazil, Dominican Republic and Venezuela. The RFLP pattern consisted of positive digestion by *Taq* I, *Sty* I and *Ssp* I. The genotypes detected in Mexico consisted mainly of standard strains such as Lukert, Edgar, STC, and CU-1. However, viruses with unique RFLP patterns consisting of digestion with *Sty* I only were also detected. This pattern did not match with any known IBDV strain.

Delaware type antigenic variant strains were detected in Colombia, Ecuador, Peru, and Venezuela. However, some strains from Venezuela exhibited a particular RFLP pattern consisting of positive digestion with *Sac* I, *Taq* I, and *Ssp* I. This unusual pattern did not match with any known reference strain and therefore was not classified by RFLP. Since the presence of the *Ssp* I site has been observed only in the vvIBDV, the genetic significance of positive digestion with this enzyme in Delaware type strains is still unclear.

The nucleotide sequence analysis of the vvIBDV strains from Brazil, Dominican Republic and Venezuela, revealed that these strains are similar to the UK-661 strain, isolated in Europe. The vvIBDV strains from Brazil and Venezuela revealed the fourteen nucleotide substitutions characteristic of vvIBDV. However, changes in two nucleotide positions were detected in the vvIBDV strains from Dominican Republic. After the amino acid sequence analysis, all the vvIBDV strains detected in Latin America showed the three amino acid residues (222 Ala, 256 Ile, and 294 Ile) that are conserved in vvIBDV strains. However, the strains from Dominican Republic showed some amino acid residues that have been detected only in strains from Asia and Africa. This suggests that parallel evolution is occurring on the vvIBDV strains detected in Latin America, but this evolution is determined by the conditions prevalent in each country.

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INFECTIOUS BURSAL DISEASE: FIELD EVALUATION ON EFFECTS OF TWO VACCINES WITH DIFFERENT LEVELS OF RESIDUAL PATHOGENICITY IN BROILERS

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ABSTRACT

Different vaccines more or less attenuated have been applied worldwide to control IBD due to very virulent virus. In the present study we compare the effects on lymphoid tissue (BF) in broilers of two vaccines provided with more or less residual pathogenicity. The bursa to body weight ratio in birds, tested weekly, has been in favor of the more attenuated vaccine (intermediate); the antigenic stimulation appeared more rapid with less attenuated vaccine.

INTRODUCTION

Infectious bursal disease (IBD) is an acute, highly contagious viral infection of young chickens due to a birnavirus (10). IBD virus (IBDV) affects primarily the lymphoid tissues, with special regard to the bursa of Fabricius (BF).

The disease has been a great concern for poultry industry due to significant economic losses worldwide for a long time (3), but particularly since the past 15 years.

In USA, it was demonstrated that from 1986 new isolates (variants) were affected by antigenic shifts (9). Whereas, in Europe, the first cases of very virulent IBDV (vvIBDV), without clear antigenic drift, were identified in 1989 (2, 16). These vvIBDV spread rather soon to all Europe (6) and after to Asia (1, 11), Africa (6), and South America (5) but not in North America (14), Australia (13), and North Europe (4).

For the control of vvIBDV, being mild vaccines ineffective (previously used with efficacy for 30 years worldwide), less attenuated strains have been introduced: so called “intermediate”, “intermediate plus” or “hot” vaccines. Studies (8, 15) showed that passages on tissue culture or in embryonated eggs had an adverse effect on immunogenic potential of a live vaccine, speculating that *in vitro* passages of this virus affect its ability to replicate in BF. More recent study (12) induced to speculate that better protection with more virulent strains of IBDV was due to more systemic antigenic stimulation on the basis of higher replication of virus in extrabursal lymphoid tissues. However, the use of less attenuated or “hot” vaccines, even with an acceptable reduction of losses, is more or less dangerous, as such strains induce immunosuppression and bring with the risk of reversion to virulence (7).

Purpose of the present survey is to compare in the field the efficacy and the side effect of two vaccines with different residual level of pathogenicity.

MATERIALS AND METHODS

Chickens: Broilers from the same breed of different age (40 to 50 wk) in their time vaccinated with live and inactivated vaccines. The maternal antibody titers by ELISA test were rather high, varying from 4561 to 10243 at hatch. All broilers were bred in field conditions and fed from the same feed mill.

Vaccines: “Intermediate” strain with a titer of 10^3 EID_{50}/dose, or “intermediate plus” with a titer of 10^2 EID_{50}/dose were administered in drinking water, respectively at 18 and 24 days or at 18 days of age in

different flocks. The birds were regularly vaccinated also against MD, ND and IB.

Experimental design: From six flocks, treated with different vaccine, ten BF were sampled at 1, 7, 14, 28, 35, 42, 49 days of age. The bursa to body weight ratios were determined. Three and ten days after vaccination, four BF of each flock were fixed in formalin, the sections stained with hematoxilin-eosin, and evaluated for histological lesions. The six flocks were also tested for IBDV antibodies at different ages using an ELISA kit (Idexx). Of other 40 flocks (20+20) the bursa to body weight ratios and antibody titers were determined at slaughter.

RESULTS AND CONSIDERATIONS

The comparison between the 3+3 flocks treated with "intermediate" or "intermediate plus" vaccine, controlled weekly, have demonstrated that the bursa to body weight ratio is apparently in favor of the vaccine with less residual pathogenicity (figure 1). However, histologic examinations of bursal lesions are in course.

The active IBD antibody movement began at 28 days of age, i.e., 10 days after a single vaccination with "intermediate plus" vaccine, reaching the top at about 49 days of age; whereas, the antibody increase in birds vaccinated with "intermediate" began about seven days later, reaching the top at the same age (figure 2). The systemic antigenic stimulation appeared higher and more rapid with less attenuated strain, due probably to its higher replication in extrabursal lymphoid tissues (12).

In regards to the results of 20+20 comparative trials, no significant differences were observed between the two kinds of vaccine, both in bursa to body weight ratio (0.47) and IBDV and NDV titers at slaughter.

In conclusion, it is our opinion that the use of stronger vaccine could be restricted only to periods of the year when breaks of immunity with "intermediate" vaccine can take place.

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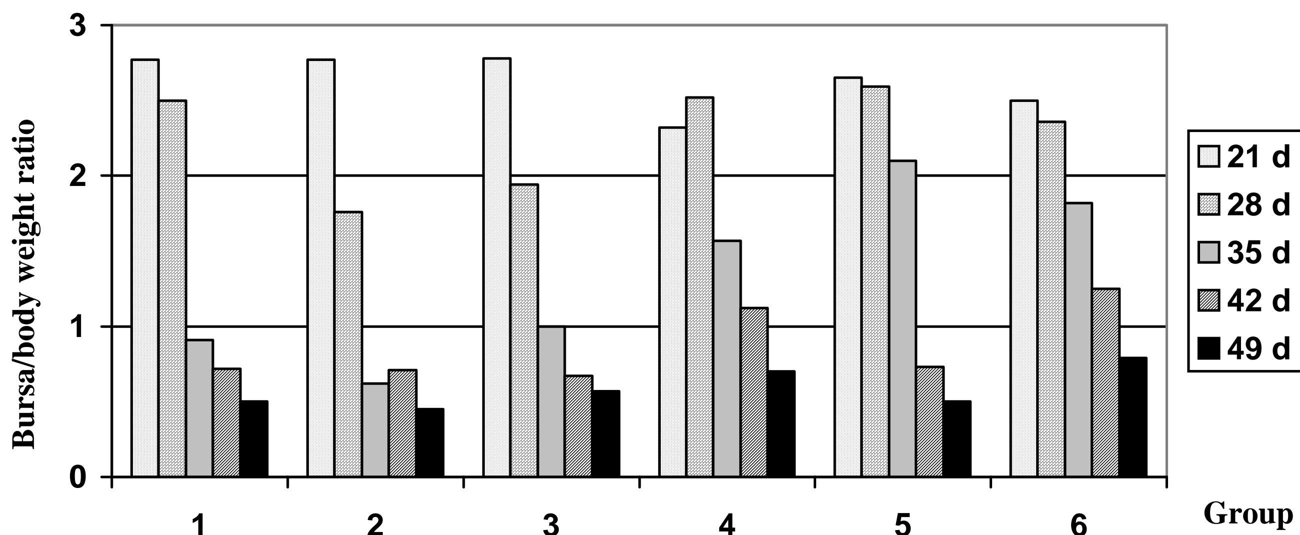

Figure 1: Bursa to body weight ratio after vaccination with intermediate plus (group 1, 2, 3) or intermediate (group 4, 5, 6) vaccine. The weight was measured at 21, 28, 35, 42, 49 days of age.

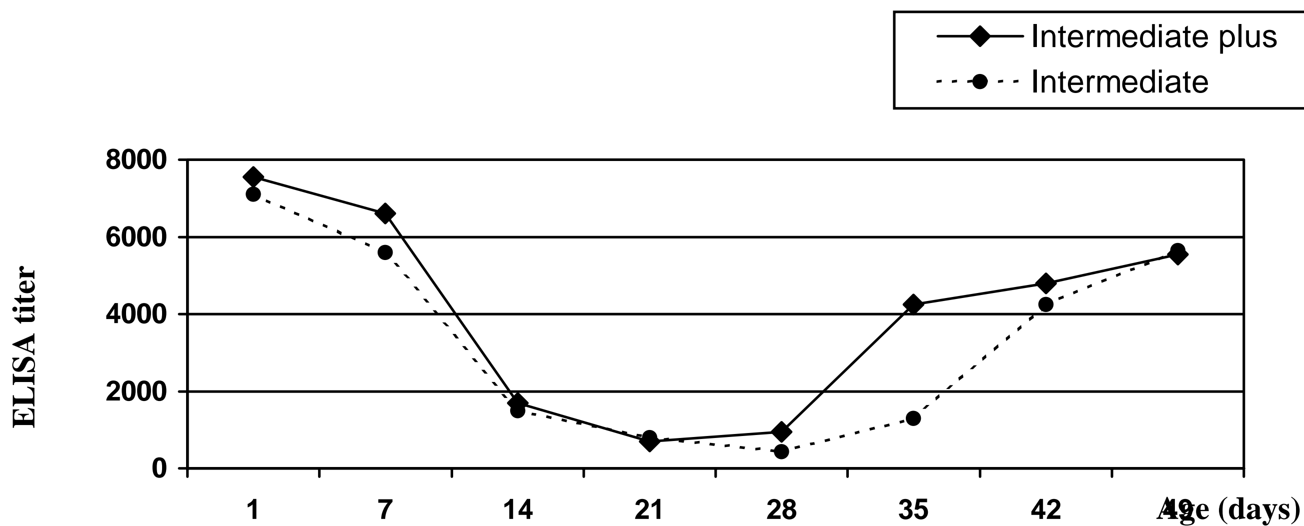

Figure 2: Graph of antibody movement in broiler vaccinated with intermediate or intermediate plus vaccine.

LARGE SCALE USE OF A RECOMBINANT VP2 VACCINE FOR THE VACCINATION AND CONTROL OF INFECTIOUS BURSAL DISEASE IN CHICKENS

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ABSTRACT

Since the appearance of the very virulent variants of iInfectious bursal disease virus (vvIBDV) in 1987, the control of the disease has been based on the development and use of new and more potent vaccines. The new live vaccines developed after the appearance of the vvIBDV were only partially attenuated and are considered in many cases as intermediate strains which are able to cross through the maternal antibody barrier at young age and vaccinate the chicks. The partial attenuation of the viruses may enable a certain level of damage to the target organs and immunosuppression (4).

New inactivated vaccines developed to confer protection against vvIBDV have been used in breeders and broilers in countries where vvIBDV is prevalent. Inactivated vaccines in breeders are supposed to provide high levels of maternal antibodies in order to protect the progeny during the first weeks of age. In order to provide adequate levels of antibodies, the new inactivated vaccines must contain very high titers of viral antigen in order to be effective.

Production of effective inactivated vaccines was based up to now on tissue culture or whole bursa infected tissue after controlled infection in chicks. This last method is considered to be highly effective, but requires the infection and sacrifice of thousands of chicks in order to produce enough antigen for mass vaccination.

In the last years attempts have been carried out to develop vaccines based on genetic engineering methods (3, 5). The first commercial subunit vaccine was developed for human use against hepatitis B virus and was expressed in *Saccharomyces cerevisae* (6).

A recombinant subunit vaccine (rVP2) against vvIBDV, was developed in a yeast expression system using the facultative methylotrpic yeast *Pichia pastoris*. The methanol metabolic pathway of *Pichia pastoris*, as in other methylotropic yeasts, involves a unique set of enzymes. In the first step of this pathway methanol is oxidized to generate formaldehyde and hydrogen peroxide, which is then decomposed to water and molecular oxygen by catalase. The oxidation is carried out by two alcohol oxidase genes AOX1 and AOX2 (2). AOX1 is the more active alcohol oxidase and may reach as much as 30% of the total protein in the cell when cultured under growth-limiting rates of methanol. This gene's promoter is utilized for expression of heterologous genes. The expression of heterologous proteins in *Pichia pastoris* is fast, simple, and inexpensive. Strong aerobic growth allows culturing at high cell densities. High levels of foreign protein expression have been shown for this vector (1).

This system of production has been used for more than five years to produce a highly effective commercial subunit vaccine containing recombinant VP2 of vvIBDV. The VP2 produced using this yeast expression system has proved to be highly immunogenic and able to provide an excellent protection against challenge with vvIBDV under experimental and large-scale field conditions.

Two field trials, for testing the efficacy of VP2 vaccine in commercial flocks under field conditions, are presented in Table 1. Results prove that broilers grown under commercial conditions and vaccinated at the age of 13 days with the recombinant VP2 vaccine are fully protected against controlled IBDV challenge at the age of five to six weeks with a vvIBDV.

This subunit recombinant VP2 vaccine has been used combined with an inactivated Newcastle disease virus or as a multivalent vaccine for breeders containing inactivated virus (Newcastle disease (ND), infectious bronchitis (IB), egg drop syndrome (EDS), and reovirus).

These combined vaccines have been used successfully under commercial conditions for more than four years in more than 300 million chickens in an area largely infected with vvIBDV. Production and use of a recombinant subunit vaccine provide many advantages over the traditional bursal tissue based vaccines.

a. No need to infect and sacrifice thousands of birds in order to produce the antigen for the vaccines.
b. No risk of contamination of vaccine with unwanted contaminants in the bursal tissue.
c. Production of vaccines with an accurate and constant amount of protective antigen (VP2).

d. The subunit vaccine can be easily modified for rapid response to viral drift by inserting the gene encoding VP2 of the new IBDV variant.

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Table 1. Field trials in broilers: rVP2+NDV vaccine compared to bursa-derived IBDV+NDV vaccine (positive)

Trial (a)	IBD Antibodies (b)				IBD Challenge (b) % protection (c)		IBD in the flock (d)		ND Antibodies (e)	
	AGP (f)		ELISA (g)							
	rVP2	positive	rVP2	positive	rVP2	positive	rVP2	positive	rVP2	positive
A	53	13	33	7	100	70	0	0	7.3	7.3
B	50	15	21	15	93	92	0	0	5.7	5.8

(a) Broilers (61000 ROSS and 46000 Anak in trials A&B respectively) were vaccinated at the age of 13 days.
(b) At the age of 5-6 weeks 20 chickens per group were bled and challenged.
(c) % Protection, measured as absense of IBDV in bursae 3 days post challenge.
(d) % Mortality or morbidity from infectious bursal disease.
(e) HI log2 (GMT). Antibodies on day of vaccination: 4.5 and 4.4 in trials A&B respectively.
(f) % Responders in AGP. Antibodies on day of vaccination: 0%.
(g) % Positive in ELISA (Idexx Antibodies test kit). Titer above 396 is defined positive. Antibodies on day of vaccination: 31% and 35% in trials A&B respectively.

THE EFFECT OF *IN OVO* IBDV VACCINATION WHEN ADMINISTERED IN MATERNAL-ANTIBODY POSITIVE CHICKENS

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One hundred forty-five (145) eggs with maternal antibodies to IBD (average ELISA titer of 11,000) were obtained from a commercial broiler integrator. Fifty (50) eggs were injected at 18 days of incubation with a full dose of an intermediate classic strain IBD vaccine (Univax-BD, Schering-Plough Corporation) and then repeatedly challenged from two to 18 days of age. Another fifty (50) were not vaccinated and served as unvaccinated-challenged controls. The remaining

forty-five (45) eggs served as unvaccinated unchallenged controls.

At hatch the vaccinated and unvaccinated-challenged groups of eggs were further divided into two subgroups of 25 chicks each. One subgroup in each group was challenged orally with the standard dose of the USDA Delaware E Strain IBD virus and the other subgroup within each group was challenged orally with a standard dose of the USDA Standard Strain IBD virus.

The birds from each group were grown in isolation units through 20 days of age. In addition to the four test groups, one group of 45 unvaccinated, MA-positive birds remained in a separate isolation unit to serve as unchallenged controls. All groups, except the unchallenged controls, were challenged orally at day 2, 4, 6, 8, 12, 14, 16 and 18 post-hatch with their assigned challenge viruses. Three bursas were harvested from each challenged group five days after each sequential challenge. Two bursas were also harvested from the unchallenged control birds every day.

In the **non-vaccinated** maternal antibody-positive birds the Delaware E strain IBD virus was detected by IDEXX RT/PCR RFLP beginning at nine days of age. The average histopathology score for the Delaware E challenged group was 2.9, based on a scale of 1 to 4 with 1 and 2 being healthy and 3 and 4 considered unhealthy. The standard strain IBD virus was detected at 12 days of age, with an average histopathology score of 1.3.

In the **vaccinated** maternal-antibody positive birds the Delaware E strain IBD virus was detected via IDEXX RT/PCR RFLP at 15 days of age. The average histopathology score for this group was 1.7. The standard strain IBD virus was not detected until 19 days of age, with an average histopathology score of 1.5.

Table 1. Univax BD *in-ovo* vaccination.

Days of challenge (standard challenge)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Univax BD group																			***1.5***	
Unvaccinated group												***1.3***								
Days of challenge (variant E challenge)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Univax BD group															***1.7***					
Unvaccinated group									***2.9***											

Maternal antibody positive birds with an average Elisa egg titer of 11000, in- ovo vaccinated with Univax BD or unvaccinated, and kept in isolation.

Virus detection and identification by IDEXX RT/PCR RFLP and bursal histology.

Numbers represent histopathology scores with 1 and 2 being healthy and 3 and 4 considered unhealthy.

CHICKEN ANEMIA VIRUS: ORAL VERSUS INTRAMUSCULAR ROUTE OF INFECTION

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ABSTRACT

The events during pathogenesis of CAV infection following intramuscular (IM) or oral inoculation were further elucidated and compared by sequential clinical, pathological, and histopathological evaluations, and by sequential determination of CAV genome concentrations in different organs. Specific-pathogen-free chickens were inoculated individually by the IM and oral routes respectively with the same dose ($2x10^6 TCID_{50}$) of CAV isolate 03-4876 at day 1 of age. Weight gain, hematocrits and samples for serology were obtained at days 7, 10, 14, 18, 21, 25, and 28 post inoculation (PI). Seven birds from each group were necropsied at days 7, 10, 14, and 28 PI and samples of thymus, Harderian gland and cecal tonsils (CT) were obtained for histopathology and CAV genome quantification by real time polymerase chain reaction. Peak CAV genome concentrations were detected in the thymus at 10 days PI in the IM and at 14 days PI in orally infected chickens. High CAV-DNA concentrations were maintained throughout the experimental period until day 28 PI despite of specific seroconversion occurring at day 14 PI. CAV was isolated from both orally and IM infected chickens at day 28 PI. Peak CAV genomes in the thymus of IM

and orally infected chickens coincided with peak lymphocyte depletion in these organs. Lymphocyte repopulation of the thymus occurred at day 28 PI in spite of the presence of the virus in the organs of both chicken groups. Despite of the presence of CAV genomes in CT, no histopathological changes could be detected. Compared with the IM route of infection, chickens infected orally did not show apparent signs of sickness. Oral inoculation determined delayed and less severe reduction of weight gain and hematocrits. Gross and histopathological changes were also less severe and delayed in the orally inoculated chickens concurrent with CAV genome concentrations increasing at a slower rate in the thymus of these chickens.

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INVESTIGATION OF SEROLOGICAL RESPONSE TO AVIAN RETICULOENDOTHELIOSIS VIRUS AFTER EXPERIMENTAL INFECTION WITH FOWLPOX VIRUS CARRYING REV SEQUENCES

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For some years it has now been known that sequences of reticuloendotheliosis virus (REV) have integrated into the genome of field isolates and vaccines of fowlpox virus (FPV) in Australia and the USA.

The size of the REV integrated fragments differs from a remnant of the Long Terminal Repeats (LTRs) to an almost complete REV provirus. By determination of the nucleotide sequence, chimeric PCR and long-range PCR, the REV-sequences were shown to be integrated into the genome and not just to contaminate the vaccines or samples. The investigation of recent FPV isolates from chicken and turkey in Germany showed that most of them had the near-full-length REV provirus integrated into their genome.

The present investigation was carried out to demonstrate the ability of one of these isolates to induce antibodies against REV in comparison to a commercial vaccine carrying only a REV-LTR remnant. Furthermore, the development of antibodies against FPV and REV was compared by ELISA.

Three groups of one-day-old SPF-chickens were infected by the wing web route as follows:

- Group 1 was infected with a German field isolate of FPV carrying a near-full-length REV-provirus with about 10^4 $TCID_{50}$/ml.
- Group 2 was infected with the same isolate with about 10^6 $TCID_{50}$/ml. The 3rd group was infected with a vaccinal strain carrying only a remnant of the LTRs of REV as recommended by the manufacture. After 5 weeks all groups were challenged i.v. with the FPV field isolate with about 10^5 $TCID_{50}$/bird.
- An additional group was kept as non-infected control.

Serum samples were tested weekly for antibodies against REV using a commercial ELISA-Kit (IDEXX). For detection of FPV antibodies, an ELISA was established and used.

The results show that the infection with the field FPV isolate carrying a near-full-length REV-provirus (groups 1 and 2) induces specific antibodies against REV. The development of the antibodies started three weeks post infection. No antibodies against REV could be detected in birds infected with the vaccinal strain (group 3). On the other hand, the humoral immunresponse against FPV was by far more pronounced in group 3.

After the i.v. challenge more chickens of groups 1 and 2 developed antibodies against FPV, but no antibodies against REV could be detected in group 3 four weeks after challenge.

All chickens of the non-infected control had antibodies neither against REV nor against FPV.

REVIEW OF CLONAL RELATIONSHIPS BETWEEN *CAMPYLOBACTERS* ISOLATED FROM POULTRY AND HUMANS IN NORTH AMERICA

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SUMMARY

Epidemiologic studies from the mid-eighties (1, 6) assign high population attributable risk factors for human campylobacteriosis to commercial chicken. More recent studies define much lower and conditional attributable risk for chicken (3, 4). In addition to epidemiologic studies, genetic subtyping can also be used as a means of assessing the possible contribution of chicken products to human campylobacteriosis by genetically comparing human case isolates to those from commercial chicken. The degree of overlap, although not proof of causation, would be an indication of the likelihood that chicken could serve as a significant source of *Campylobacter* infections in humans. Molecular methods employed such as PCR-RFLP, ribotyping, RAPD, PFGE, AFLP, and MLST have varying levels of discriminatory power and thus provide differing levels of information about the genetic "relatedness" of compared isolates. As discriminatory power of genetic methods increase, the population "overlap" decreases because more sensitive methods are better able to distinguish two isolates as "different". Of the enteric pathogens *Salmonella* and *E. coli* are relatively easy bacteria to type because they are genetically stable organisms. *Campylobacter* on the other hand is an organism with widely varying levels of genetic instability. Some strains are quite unstable while others are very stable. Levels of genetic instability and discriminatory power must be carefully considered when drawing conclusions from the results of molecular subtyping methods (5, 9, 13).

OVERVIEW

The discovery of the genetic instability in *Campylobacters* is a relatively recent finding (5, 13). This genetic instability is thought to be the result of environmental stress "bottlenecks" in which *Campylobacters* are highly pressured for genotypes which are able to survive harsh environments followed by the finding of new niches where these selected genotypes can amplify and disseminate (13) thus contributing to large amounts of genetic diversity within the genus. *Campylobacter* is known to be a naturally transformable organism (13) and one in which inter and intragenomic recombination may occur (5) at least at the flagellin gene locus. It is also known that deletions and other internal chromosomal segment rearrangements can obscure the interpretation of PFGE analysis (9, 13).

Most of our knowledge comparing population overlaps of *Campylobacters* causing disease in humans with animal and environmental reservoirs come from European studies (9). However, it may be misleading to attempt to extrapolate the findings of European studies to the US situation mainly because the epidemiology of *Campylobacter* can be different in different countries. For example, campylobacteriosis has decreased in the US for the sixth consecutive year (7) while in Europe incidence is roughly two-fold higher and in many countries increasing (9). In order to consider more accurately the contribution of chicken products to campylobacteriosis in the US it is instructive to examine in detail the three recent studies reported from North America (2, 8, 12). Of these, two studies were conducted in the U.S. (2, 12) and one in Canada (8).

The study of Nadeau *et al*. (8) employs biotyping & PFGE for isolate analysis, and compares human and poultry isolates from the St-Hyacinthe region of Quebec over approximately the same time frames. A particular drawback of the study is that poultry samples are derived from live animals at processing and not from processed poultry. Newell *et al*. have shown that strains which survive processing may only represent a small subset of those found in live animals (10). However, strains present on processed poultry are of most interest since these are the strains most likely to reach the consumer. At a 94% homology level the authors estimate that there is a 20% potential overlap between human and poultry *Campylobacters* which are indistinguishable or genetically related. This leaves roughly 80% of human cases without a hypothesized reservoir source.

The study of Smith *et al*. (12) uses PCR-RFLP of the fla-A gene, to analyze human and poultry isolates collected from presumably different areas and over somewhat different time spans. Retail poultry isolates collected between September 8 and November 3 of 1997 in the Minneapolis- St. Paul area were compared with human isolates from the entire state of Minnesota

during 1997. No linkage in time and space between case isolates and poultry products was made by the authors, yet they allude to an "association" between fluoroquinolone resistant (FQ-R) *Campylobacter jejuni* subtypes on chicken products and those found in human cases based on the fla-A patterns. Oddly, group comparisons were discussed purporting more frequent associations between chicken FQ-R types and domestically acquired human FQ-R types even though FQ-S types predominated in both chicken products (86% of total) and human isolates (97% of total for 1998). First, if biologically meaningful relationships were identified, and poultry products were a plausible source, one would expect to find "real" associations first among the human and poultry FQ-S *C. jejuni* populations which represent the largest majority of both populations. Second, it is not logical to conclude that poultry was responsible, based on genetic typing, for the human campylobacteriosis cases when most of the poultry isolates were collected several months after the human infections occurred. A recent study from Italy (11) comparing the highly discriminatory AFLP method to fla typing establishes that fla-A typing is epidemiologically unreliable when comparing isolates separated in time and space. This is consistent with many other reports (5, 9, 13). As a result, it is not possible to glean substantial overlap information from this study.

The study of Dickins *et al*. (2) is perhaps the best and most recent study comparing human and poultry *Campylobacters*. This study uses PFGE of human case isolates (collected over a two year period) and compares typing results with *Campylobacters* obtained from commercial chicken products (collected over a one year period) in the Little Rock, AR area. Both groups of samples have temporal and geographical overlap potential. This study finds 4 human PFGE types (using two restriction enzymes) out of 54 human cases samples that are identical to patterns found in commercial poultry samples. This represents a strain overlap of approximately 7%. Unfortunately, no other potential reservoirs were examined and 93% of the human cases are left without a plausible reservoir source.

CONCLUSION

When only molecular typing studies in North America are considered the "overlap" of human and poultry *Campylobacters* appear to be 20% or less. This finding is in agreement with recent epidemiological studies (3,4) and supports the contention that chicken products in the U.S.(and possibly Canada) are less of a potential reservoir for *Campylobacter* than previously thought. Because other potential reservoirs were not sampled in these studies and because temporal and geographical relationships are not clear or unreported, causal conclusions must be withheld (e.g. "common source" infection of both chickens and humans cannot be ruled out). In order to have any clear idea of the true reservoir sources for human campylobacteriosis larger sampling studies from all possible reservoir sources would need to be included and analyzed together. This should include humans and human effluent waste waters as well as those from confined animal facilities, processing plants, and pastureland irrigated with reclaimed waters. Pets, organic vegetables & juices, and recreational reservoirs frequented by the public would also be important previously untested potential reservoirs that should be examined. It is hoped that this review will facilitate and stimulate unbiased investigational work to advance our state of knowledge regarding the relative contribution of all potential sources of human campylobacteriosis.

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BROILER CHICKS INOCULATED EXPERIMENTALLY WITH *CAMPYLOBACTER JEJUNI*

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ABSTRACT

One hundred and ninety one-day-old broiler chicks were obtained from a local hatchery. The chicks were necropsied and their intestines were cultured for the presence of *Campylobacter*. Serum samples were tested for MG, MS and *Salmonella pullorum*/*typhoid* antibodies. The remaining 180 chicks were subdivided into nine groups of 20 chicks and housed in Horsfall units. Commercial broiler feed and water was provided *ad libitum*. Four groups of chicks were inoculated individually with one of the *Campylobacter* spp. containing 0.5 ml of 1×10^2 CFU of *Campylobacter* by crop gavage at 9 days of age. The other four groups of chicks were inoculated similarly with the same *Campylobacter* spp., but the inoculum contained 0.5 ml of 1×10^4 CFU of the *Campylobacter* spp. One group was kept as an uninoculated control.

At 5, 12, and 19 days post inoculation, four chicks were collected at random from each treatment group. These chicks were euthanized, necropsied, and the intestinal tissues were cultured for *Campylobacter* enumeration and histopathology. All chicks were weighed at 14, 21, and 28 days of age and statistical analyses performed. The study was terminated at day 28.

Reduced body weights were not observed at different weighing intervals in the inoculated groups as compared to controls. Mortality was not observed in any of the inoculated groups. Results of *Campylobacter* enumeration from the ceca were 2 to 3 logs higher as compared to the upper and mid intestine samples. *Campylobacter* was not isolated from the intestines of day old broilers or the uninoculated controls at different intervals.

SUBPOPULATION BEHAVIOR OF EGG-CONTAMINATING *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS AS DEFINED BY LPS MASS

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ABSTRACT

Background: *Salmonella enteritidis* contaminates the contents of the hen egg and it is thus a major world-wide food safety problem. High-molecular-mass LPS (HMM LPS) produced by some isolates alters avian reproductive tract biology. Cooperation between orally invasive and parenterally-adapted subpopulations appears to be required to achieve high-incidence egg contamination. Objectives were to determine if parenterally adapted subpopulations that make HMM LPS and have reproductive tract tropism emerge during the infection pathway to the egg. Methods: Neutral sugar compositional data were derived by gas chromatography of derivatized LPS obtained from isolates cultured from experimentally infected hens, from the spleens of naturally infected mice, and from United Kingdom isolates. Statistical analyses were performed as indicated. Results: The probabilities that avian isolates produced HMM LPS suggested that U.S. mouse isolates were most like U.S. avian intestinal samples, whereas UK isolates were most like avian reproductive tract and egg isolates. Non-reproductive tract organ isolates had significant loss of O-chain. Isogenic isolates with different abilities to make biofilm and to be orally invasive produced different O-chain structures at 25 but not at 37°C. Hens infected at a 91:9 positive/negative biofilm colony phenotype ratio yielded only the negative phenotype from eggs. Conclusions: These results indicate that the hen applies stringent selection pressure to subpopulations of *Salmonella enteritidis*. The avian cecum was an early environment that supported emergence of strains producing HMM LPS. These results suggest that diet and other factors that alter gut physiology could be manipulated to specifically reduce egg contamination as compared to just achieving reduction in intestinal colonization.

INTRODUCTION

Salmonella enterica serovar Enteritidis (*S. enteritidis*) is the leading cause of food-borne salmonellosis worldwide, in part because it is the only one of more than 2,000 serotypes that efficiently contaminates the hen egg and causes human illness (1,2). It is important to determine differences between *S. enteritidis* and other *Salmonellae*, because this information could help reduce egg contamination, specifically, as compared to carcass contamination. Egg-contaminating *S. enteritidis* is predominantly clonal (3-7), but it nonetheless generates substantial phenotypic variation that alters the incidence of egg contamination in infection models. Chemotyping of lipopolysaccharide (LPS) O chain is a sensitive method of phenotypic analysis that combines stoichiometry with statistical analysis to produce clusters of data that correlate with LPS O-chain structure(8). Chemotyping has shown that *S. enteritidis* efficiently produces high-molecular-mass (HMM) LPS O chain, whereas *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) does not (9,10,11). Infection of hens with a high systemic dosage of a strain of *S. enteritidis* that produces HMM LPS results in regression of the reproductive tract and dramatic loss of production, whereas birds infected with *wzz* mutant *S. enteritidis* remain in production. Thus, HMM LPS is a molecule that influences avian reproductive tract biology in a remarkable way (12). The effect of HMM LPS at low dosage is to mitigate signs of disease in hens, but not

egg contamination (12, 13). Previous research has shown that the best source for isolates that efficiently produce HMM LPS is the egg (8). However, strains recovered from the egg are at the end point of selection within the chicken; thus, it is important to determine more precisely when egg-contaminating strains first appear in the hen.

APPROACH

Source of isolates. *S. enteritidis* was cultured from the intestine (pooled duodenum, jejunum, and ileum), paired ceca, organs (pooled liver, kidney, and spleen), reproductive tract tissue (ovary and oviduct), separated egg fractions (yolk and albumen), and whole egg obtained from experimentally infected hens using published techniques.

LPS analysis. LPS O chain was analyzed by gas chromatographic analysis of derivitized alditol acetates to determine the yield of rhamnose. Standard bell curves were constructed, using fifth-polynomial curvilinear analysis, from average rhamnose yields and standard deviations to visualize subpopulations. To further refine details of the infection pathway leading to egg contamination, LPS O chains from pairs of biofilm-positive and -negative isolates from the United Kingdom were analyzed at permissive and nonpermissive temperatures of 25 and 37°C, respectively. Samples were run on a 25-m silicone midpolar column (Quadex, Cat. No. 007-17-25W-0.25F) at an initial temperature of 150°C for 2 min, which was increased at a rate of 4°C/min and held at 260°C for 10 min or until the heptose peak was present. Smooth isolates are classified as those that produce HMM and LMM LPS. Isolates with yields of at least 12 μg of rhamnose/100 μg of LPS produce ≥50% HMM LPS, whereas those with between 4 and 12 μg of rhamnose/100 μg of LPS produce more LMM than HMM LPS. Isolates that yield <4 μg of rhamnose/100 μg of LPS no longer react well with group D1 serotype reagents (also called somatic O antigen), and thus, they are described as being rough.

Statistical analysis. Rhamnose yields that were 3 standard deviations (SD) from the average, as well as the average itself, were used to generate a bell curve by application of the fifth-polynomial differential (Slidewrite version 6). A fifth-polynomial curve fit equation was applied to generate a standard bell curve to show the range of data that might be encountered for this one isolate in any particular evaluation. The area under the curve thus reflects the average of the data set, as well as the deviation that can be expected to occur within the subpopulation.

Animal infection studies. SPF leghorn hens between 25 and 55 weeks of age were contact infected by first injecting 4 to 6 hens out of 24 per experimental group with 10^6 CFU of *S. enteritidis* intravenously. The strains used to challenge hens, namely, SEPRL 22023, 21000, and 20127, are isogenic as determined by internationally accepted standards using two-enzyme ribotype analysis, (7) and they are all phage type 13a (data not shown). The phenotypes of these three strains in regard to their ability to produce HMM LPS O chain (HMM) and biofilm (bf) were as follows:

- 22023, HMM^+ bf^+
- 21000, HMM^+ bf^-
- 20127, HMM^- bf^-.

Results (Figure 1A and Table 1. The avian cecum was the first anatomical site under evaluation that where *S. Enteritidis* subpopulations emerged in the hen to produce HMM LPS. Reproductive tract organs and eggs selected for gain of LPS O-chain mass. Non-reproductive tract organs selected for loss of LPS O-chain mass. The intestine selected for a surprisingly homogeneous population that produced a low-molecular-mass (LMM) O-chain typical of *S. Typhimurium*. Organs and yolk supported emergence of subpopulation diversity, but isolates lost O-chain in organs, whereas there was a gain in O-chain in any niche associated with reproductive tract organs. The rank for isolates producing HMM LPS was, from least to greatest: non-reproductive tract organs < intestines < cecum < ovary, oviduct < albumen < yolk < whole egg. The whole egg is an environment that polarized *S. enteritidis* isolates into subpopulations that were similar to avian intestinal isolates and those that maximized HMM LPS production. The spleen of the naturally infected house mouse also polarized subpopulations of *S. enteritidis*, as did whole egg. High incidence egg contamination following contact infection was associated with the presence of two subpopulations, one which produced biofilm but no HMM LPS and one that produced HMM LPS but no biofilm.

SUMMARY OF RESULTS

The anatomical juxtaposition of two egg microenvironments that both support production of HMM LPS, namely, albumen as a highly selective environment and yolk as an environment that supports diversity, might contribute in some way to enhancement of virulence for humans because we often eat whole eggs.

The mouse generates an unusual degree of *S. enteritidis* subpopulation diversity and it may constantly seed the henhouse environment with orally invasive phenotypes.

Infection of the hen that results in egg contamination appears to follow a general concept in epidemiology, which states that major changes in

patterns of disease occur when the mucosal invasiveness of a pathogen increases. This is because wild-type *S. enteritidis* mixed with the biofilm phenotype produced a high incidence of egg contamination, whereas the wild type alone did not.

LPS characteristics of biofilm forming and wildtype *S. Enteritidis* are temperature dependent, as is colony phenotype.

These results strongly support the concept that a helper phenotype that is itself not recovered from eggs is an important part of the infection pathway resulting in egg contamination.

DISCUSSION

The paired ceca of poultry appear to be an early interface between environment and host that correlates with emerging subpopulation diversification. Certain anatomical aspects of the digestive tracts of farm animals distinguish their abilities to efficiently convert fibrous plant materials to energy. In brief, all farm animals have some anatomical site that facilitates the conversion of feedstuffs into volatile fatty acids, which are then converted into glucose for metabolic processes that support efficient production of food and fiber. Poultry have paired ceca, the horse has a single cecum and a fermenting large intestine, and the true ruminants have the four-compartment stomach. Without these structures and an associated enhanced ability to digest a range of feedstuffs, it is unlikely that modern farm animals could reach the level of production that they have. The pig is the farm animal with a monogastric digestive tract that most closely resembles that of humans, but even it has a well-developed cecum compared to the vestigial human organ (the appendix). If gut physiology is an important reason why *Salmonellae* persistently colonize farm animals, then the cecal physiology of the hen may play a specific role in supporting the emergence of *S. enteritidis* with tropism for the reproductive tract. There is some molecular evidence that the cecum supports the growth and colonization of subpopulations of *Salmonella* that vary in LPS O-chain composition, whereas the intestine favors smooth strains (14). Gut physiology can be manipulated, and many types of biological controls and diets have been designed with the idea of excluding pathogenic *Salmonellae* from the gut (15-21). Refinement of these approaches to suppress the emergence of subpopulations of *S. enteritidis* that have tropism for the reproductive tract of the hen might further reduce the number of contaminated eggs that reach the market. Differences in nutrient sources, molting practices, and other dietary management practices among continents and regions could have a substantial impact on subpopulation dynamics. In regard to *S. enteritidis*, additional research on how avian cecal biology alters subpopulation diversification will be required in order to develop application from theory.

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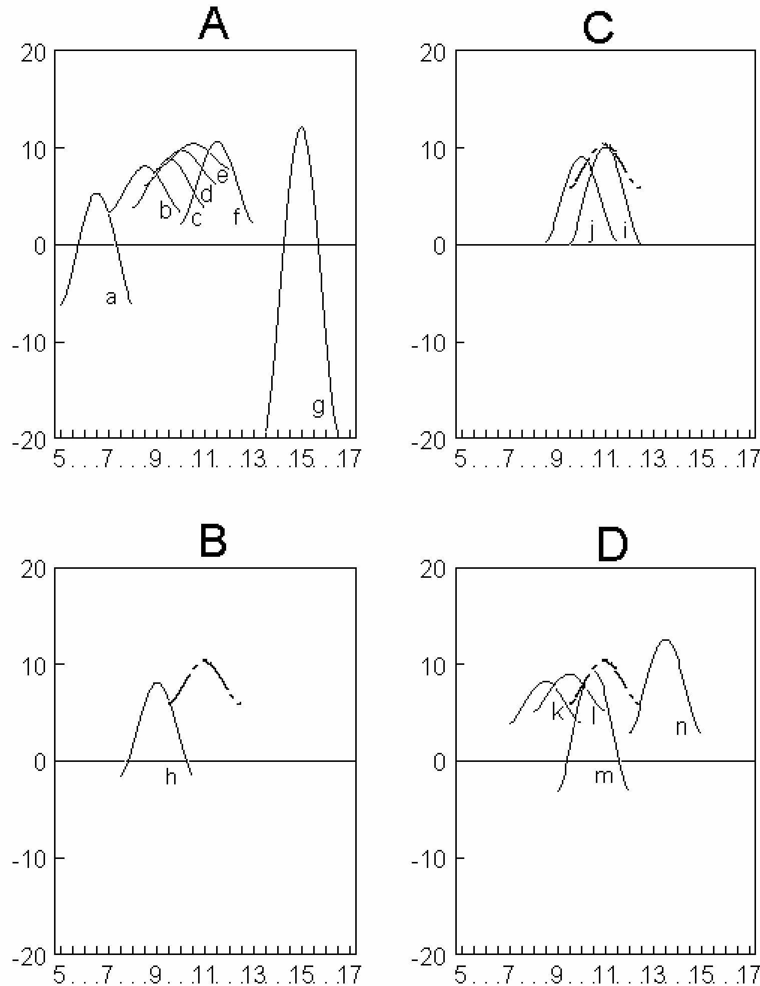
TABLE 1: Characterization of *Salmonella enteritidis* subpopulations by LPS chemotype

								Student's *t* test *P* value		
Source	Fig. 1 curve label	No. of isolates analyzed	% HMM LPS	% LMM LPS	% rough	Average yield rhamnose (ug /100ug LPS)	SD	Whole-egg comparison[a]	*wzz* mutant comparison	No. of subpopulations
Organ	a	6	0	66.7	33.3	6.43	4.21	0.056*	0.010*	2
Intestine	b	11	0	100.0	0	8.57	1.77	0.031*	0.007*	1
Ceca	c	6	0	100.0	0	9.37	1.88	0.126	0.082	1
Ovary/oviduct	d	5	0	100.0	0	10.12	1.31	0.180	0.238	1
Albumen	e	5	0	100.0	0	10.70	0.95	0.207	0.468	1
Yolk	f	5	20.0	80.0	0	11.52	3.11	0.256	0.288	2
Whole egg	g	6	33.3	66.7	0	15.20	11.51		0.229	2
Mouse spleen	h	14	14.3	78.6	7.1	9.01	3.53	0.039*	0.101	3
UK all, 25°C	i	10	20.0	80.0	0	11.04	3.67	0.152	0.424	2
UK all, 37°C	j	10	10.0	90.0	0	9.93	3.22	0.095	0.258	2
UK bf+, 25°C	k	5	0	100.0	0	8.58	1.57	0.120	0.019*	1
UK bf-, 37°C	l	5	0	100.0	0	9.38	1.42	0.149	0.075*	1
UK bf+, 37°C	m	5	40.0	60.0	0	10.48	4.54	0.209	0.436	2
UK bf-, 25°C	n	5	60.0	40.0	0	13.50	3.56	0.382	0.042*	2
wzz mutant		8	0	100.0	0	10.77	1.65	0.150		1

[a] Probability values < or = 0.075 indicate significant difference (*).

Figure. 1. Curvilinear analysis of *Salmonella enteritidis* subpopulation diversity. All curves were generated by application of 5th polynomial analysis of the average yield of rhamnose +/- 3 stddev as described in Materials and Methods.



Curve set A: Avian isolates, as recovered from: (a) organs (pooled kidney, liver, spleen); (b) intestine (pooled duodenum, jejunum, ileum), (c) ceca; (d) ovary, oviduct; (e) albumen; (f) yolk; (g) whole egg. Cultures were grown at 37°C as otherwise described in text. The control curve generated for *wzz S. enteritidis* shown in sets B-D as a broken line overlaps with (e) albumen in curve set A.

Curve set B: (h) Mouse spleen isolates, as compared to curve from (broken line) *wzz S. enteritidis*.

Curve set C: Overall average for (i) United Kingdom isolates, grown at 25°C; (j) United Kingdom isolates, grown at 37°C. (broken line) *wzz S. enteritidis*.

Curve set D: United Kingdom isolates, grown at temperature indicated, and divided by phenotype as follows: (k) biofilm+, 25°C; (l) biofilm–, 37°C; (m) biofilm+, 37°C; (n) biofilm-, 25°C. See Table 1 for accession and phenotype information for United Kingdom isolates. (broken line) *wzz S. enteritidis*.

EFFICACY OF A LIVE *SALMONELLA TYPHIMURIUM* VACCINE GIVEN BY DIFFERENT ROUTES AND THE INFLUENCE OF SAME-DAY ANTIBIOTIC ADMINISTRATION

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INTRODUCTION

There are three live *Salmonella typhimurium* (ST) vaccines currently licensed for chickens. All three vaccines are given by mass application, either by coarse spray or drinking water application. The first dose is usually applied by spray either in the hatchery or within the first few days of life. Because live bacterial vaccination by injection is not unprecedented in poultry (4), this study was conducted to see if wing web or subcutaneous injection of a live ST vaccine would elicit *Salmonella* protection comparable to oral vaccination. And because antibiotics are commonly given with Marek's vaccine, a group receiving both live vaccine and antibiotics was included. Fort Dodge's Poulvac ST was the vaccine used in this study. Its attributes have been described previously (1, 2, 3).

MATERIALS AND METHODS

Day of age SPF pullets were housed in Horsfall isolator units until the termination of the study at 49 days. Groups of 25 birds each were given a single dose of live ST at seven days of age, as follows: 1) drinking water, 2) drinking water along with a full dose of Naxcel given subcutaneously (subQ), 3) wing web injection, 4) subQ injection, and 5) no vaccination. At 42 days of age, all birds were challenged by 1.0 ml oral gavage with a virulent *S. typhimurium*. One week later all birds were sacrificed and ceca and internal organs were cultured for the presence of the challenge organism. Organ pools consisted of liver, spleen, and kidney. Statistical analysis was done using the Chi-square test ($p<0.05$).

RESULTS

Challenge controls gave an *S. typhimurium* challenge organism recovery rate of 24/25 from ceca and 23/25 from internal organs. The drinking water route of vaccination gave protection as measured by significantly lower re-isolation rates from the ceca (13/26) and organs (10/26). The drinking water route plus subQ Naxcel gave significant protection in the organs (10/25) but only modest *Salmonella* reductions in the ceca (21/26). The wing web and subQ routes of vaccination both showed significant ST protection of the internal organs with re-isolation rates of 4/26 and 7/25 respectively, but only the wing web route gave significant cecal protection as well (17/26).

DISCUSSION

The numerical improvement of parenteral vaccination over drinking water in the internal organ recovery rate suggests there is a potential for better systemic immunity by injection. Wing web injection elicited a better immune response than subQ, especially locally in the cecum. While results showed that wing web and drinking water administrations of Poulvac ST both resulted in significant protection of organs and ceca, the numerical differences may suggest that oral vaccination results in better gut immunity and wing web injection offers better systemic immunity. Future studies could be conducted to see if a combination of both oral and wing web vaccinations resulted in the most optimal live vaccine protection. Finally, the safety and compatibility of wing web administration with other vaccines should be confirmed.

Simultaneous injection with antibiotics at the time of water vaccination did not affect systemic immunity as measured by organ protection, but cecal protection was indeed diminished by five weeks post vaccination. Because Poulvac ST calls for a booster vaccination two weeks after the initial spray vaccination, the significance of this finding is not known. However, programs which rely on a longer interval between the first two vaccinations should consider the effect that hatchery administered antibiotics may have on the first ST vaccination and, thus, the overall vaccination program.

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INVESTIGATION INTO THE CAUSE OF EXCESSIVE EARLY MORTALITY IN BROILERS

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ABSTRACT

An integrated poultry company reported an increase in seven-day mortality from less than 1% to greater than 2% complex wide. Mortality tended to be highest in the first three days. Hatchery and on farm evaluations were performed to determine the cause of elevated mortality. Hatchery evaluation did not reveal any abnormalities in sanitation, egg pack, or chick quality. Clinical examinations were performed on two farms. Farm A had two-day-old chicks with a total mortality of 1.5%. Farm B had four-day-old chicks with a total mortality of 3%. Clinical signs and necropsy lesions were similar on each farm. Approximately 1% of the chicks were depressed, pale, and unresponsive to external stimuli. Internal examination of moribund and dead chicks revealed pale internal organs and watery blood. The intestines were thin walled and distended with gas. Most chicks had feed in the crop. Large multifocal ulcers were present in the gizzard and the koilin lining was dark. Two chicks had hemorrhage on the mucosal surface of the proventriculus.

A complete set of tissues was collected for histopathology, liver was collected for toxicology, and feed samples were taken for analysis. A feed toxicity was suspected based on clinical signs and necropsy lesions but the feed analysis was normal. Microscopic lesions were present in the proventriculus, gizzard, liver, and kidney. Multifocal mucosal ulcers were present in the proventriculus. The gizzards had severe diffuse fragmentation of the koilin lining with extension to the underlying mucosa resulting in heterophilic infiltration. There were diffuse kupfer cell hyperplasia and hypertrophy with erythrophagocytosis in the liver. The kidneys had a severe accumulation of hemoglobin in the proximal tubular epithelial cells and calcium urate uroliths in the medullary collecting ducts along with heterophilic infiltrates and epithelial ulceration.

Toxicology testing was performed on the livers to measure copper, selenium, and zinc levels. Selenium and zinc levels were within the normal range but copper levels were elevated. Normal liver copper levels are between 3 and 15 ppm. Liver levels of copper between 20 and 150 ppm are considered toxic. Liver copper levels measured 66 ppm in samples taken from the Farm A and 88 ppm in samples taken from Farm B. A diagnosis of copper toxicity was made based on toxicology and histopathology results. Copper sulfate is commonly used in broiler feed. It comes in two forms: powder and flake or granular. The granular form can cause gastrointestinal burns or ulcers especially in the proventriculus and gizzard. The level will be toxic if it is over 2 lbs/ton of feed. Toxic levels don't have to be present in the feed to cause problems though. If the granules are large enough, broilers will preferentially pick them out resulting in gastrointestinal ulcers and mortality. Chicks have a greater tendency to do this so granular copper sulfate is not recommended for starter diets. Granular copper sulfate was used in the diets of these birds and was responsible for the elevated early mortality.

VETERINARY DIAGNOSTIC SERVICE AND VILLAGE ANIMAL HEALTH WORKERS IN CAMBODIA

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SUMMARY

This presentation will report the serious destruction of veterinary services following the Vietnam War during the Pol Pot government in Cambodia. This led to heavy losses of livestock, especially water buffalo, from hemorrhagic septicemia for which there was no vaccine. Veterinarians from Heifer International and other non-government organizations received permission to re-establish a vaccine facility. A laboratory was constructed in a 40-foot shipping container in Singapore and shipped to Phnom Penh by barge. Because it was a "ready-to-go" laboratory, it was immediately operational.

Comments will also be made about the village animal health workers.

GAME BIRD HEALTH AND MANAGEMENT EDUCATION IN PENNSYLVANIA: PAST AND PRESENT

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Game bird breeding, propagation, and hunting on private preserves have had a long history in Pennsylvania. An organized Game Bird Breeders Association has been in existence since 1941. This association has had close ties with the Penn State Cooperative Extension Departments of Poultry and Veterinary Science, the Pennsylvania Game Commission, the Poultry Council of Penn Ag Industries, and the Pennsylvania Animal Diagnostic Laboratory System. These positive interactions have resulted in continued growth and profitability for this group which currently boasts over 300 members and several farms producing over 250,000 birds/year. The total number of game birds in Pennsylvania is estimated in the millions, with over 400 operating permits issued by the Pennsylvania Game Commission.

The *Pennsylvania Game Bird Bulletin* had its first issue in 1967 and is still in existence today. The first editor was Mr. Lynn Laudenslager of Mahantongo Game Farms. Advisors for the *Game Bird Bulletin* included Dr. L. Dwight Schwartz, who wrote a manual on game bird diseases in 1995. Debra Trace, of Trace Pheasantry took over from Mr. Laudenslager and continued a high quality bulletin for seven years. The current editor of the *Game Bird Bulletin* is Dr. David Kradel, a veterinary consultant. This newsletter has provided useful information to game bird growers both in Pennsylvania and the US and continues to be quoted liberally by other similar publications.

The Department of Poultry Science has provided in depth continuing education programs for game bird producers with an annual conference and short course. The first conference, titled: Game Bird Production and Management of Shooting Preserves was held in State College, PA in 1968. Speakers in poultry science, agricultural engineering, veterinary medicine, the game bird industry, and other disciplines have provided much needed information. The meeting is usually capped by an afternoon "necropsy session" conducted by veterinarians from the three diagnostic laboratories in Pennsylvania where growers learn the basics of disease recognition. While initiated to serve the needs of the local industry, the meeting now hosts clientele from New England to the Midwestern States. The most recent meeting had 144 people in attendance.

Biosecurity has always been a big concern for the Pennsylvania Poultry Industry, particularly due to the numerous avian influenza outbreaks that have occurred in this state. They have recognized the importance of working with and cooperating with the sizable game

bird industry. When the USDA produced its education video titled: "Biosecurity in the Poultry Industry" in 1995 a special section of the tape discussed biosecurity in the game bird industry. This segment was filmed at a large game bird operation in Pennsylvania and demonstrates the commitment of this industry to participate as a team player in avian disease control.

BOTULISM IN PHEASANTS, CHUKARS, AND QUAILS

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ABSTRACT

In June 2003, the California Animal Health and Food Safety Laboratory (CAHFS), Fresno Branch, received a series of cases from one commercial ranch that raised pheasants, chukars, and quails for hunting clubs. Birds ranged in ages from 5-10 weeks old. The birds were presented with a history of high mortality within 12-24 hours after displaying signs of leg weakness, limp necks, and prostration. The illness was first reported in pheasants and spread rapidly to other houses and affecting the quails and chukars. In pheasants, the mortality reached 100% in two days, and the chukars stayed morbid the longest with the whole flock eventually succumbing to death after four weeks.

Except for mild catarrhal enteritis observed in all three bird species, lesions at necropsy and histopathology were inconsistent and non-specific in all three species. Coccidial oocysts were observed by direct microscopic examination of intestinal scrapings from pheasants. Fly maggots were found in the gizzard of one pheasant. Toxicities due to heavy metals, ionophores, and sodium, and vitamin E deficiencies were ruled out by feed, water, and tissue analyses. All birds tested were serologically negative for mycoplasma, avian influenza, and Newcastle disease (ND). RRT-PCR for END was negative, direct electron microscopy for viral particles was negative, and virus isolations for APMV-1 were negative. Bacterial isolation from the intestines was positive for *Salmonella reading* in some of the pheasants and chukars.

Several attempts were made to detect the botulinum toxin via the mouse bioassay using sera, intestinal and gizzard contents, soil, and water. A positive mouse bioassay was seen in only one sample of pheasant gizzard contents, two serum samples from affected pheasants, and one soil sample collected from a pheasant flight house. Three other serum samples from pheasants tested positive for type C botulinum toxin using the ELISA. Botulinum toxin was never detected from any samples submitted from the chukars and quails. There were at least three visits made to the farm at the height of this crisis. Observations and information garnered from the owner led us to believe that the index case occurred in a pheasant flight pen from a dead carcass that was not immediately removed, thereby allowing maggots to proliferate. It was only a matter of hours when flies and people spread the toxin to nearby houses making the case full blown.

This case emphasizes the well-publicized difficulty in detecting botulinum toxin, not only in mammalian, but also in avian species (2-4). Since inability to demonstrate the toxin does not always rule out the diagnosis of botulism, clinical signs and history of exposure to dead carcass, maggots, and spoiled feed have been valuable tools in reaching a diagnosis. The LD_{50} for type C1 botulinum toxin has been determined in some gallinaceous birds using the mouse-lethal-dose-fifty (MLD_{50}) (1). In pheasants it is 60 MLD/kg, in turkeys 320 MLD/kg, in peafowl 2,700 MLD/kg, and in chickens 16,000 MLD/kg. Values for the quail and the chukars have not been reported.

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(A case report will be submitted to *Avian Diseases* for publication.)

UPDATE ON THE DEVELOPMENT OF A PROPOSED PLAN FOR THE CONTROL OF LOW PATH H5/H7 AVIAN INFLUENZA

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INTRODUCTION

Until 1983 avian influenza was a disease primarily of academic interest and of concern to those involved in prevention of foreign animal diseases in the United States. Fowl plague had not occurred for more than 50 years and infections with avian influenza viruses were not well recognized by the poultry industry.

The outbreak of low pathogenic avian influenza H5N2 in Pennsylvania in 1983 changed the way that the U.S. government, the poultry industry and academics thought about avian flu. The outbreak began in April as a low pathogenic infection causing respiratory disease, declines in egg production and little to moderate mortality in most of the 23 flocks that I diagnosed over the next six months. That changed suddenly when, in October, two flocks were presented to the lab on the same day with a history of up to 90% mortality in 10 days and showed the classical lesions of fowl plague that were described by Dr. Evan Stubbs in Pennsylvania in 1925. There were several very important lessons learned from this outbreak. First, low pathogenic avian influenza infection of commercial poultry was not a reportable disease in many states and the United States Department of Agriculture (USDA) did not have a program or the authority to control low pathogenic avian influenza or to spend money to do it. Second, for the first time scientists saw the low pathogenic virus change to a high pathogenic virus and could now study both viruses to determine how they differ. Third, export markets that were becoming so important to the economy of the poultry industry were severely affected.

Over the intervening years, the export markets have become much more important to the poultry industry and even though low pathogenic avian influenza virus infection in commercial poultry is not a reportable disease to the Organization International des Epizooties (OIE), many countries illegally close the markets within hours or days of an outbreak. An increased interest in influenza viruses and improved diagnostic efforts have resulted in the recognition of many more low pathogenic infections in commercial and non commercial poultry. Some of these have resulted in widespread infection of many flocks that experienced significant clinical effects and mortality. Efforts to control and eradicate these low pathogenic outbreaks are costly and time consuming. We continue to see the immediate closing of the foreign markets to these outbreaks and the severe economic losses that come with it.

As a result of the low pathogenic H7N2 avian influenza outbreak in Virginia in 2002, the USDA, Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS) requested that the United States Animal Health Association (USAHA) Committee on Transmissible Diseases of Poultry and Other Avian Species convene a special session prior to the Biennial Conference of the National Poultry Improvement plan (NPIP) in San Antonio, Texas to provide stakeholder input on the future approach to low pathogenic H5/H7 avian influenza virus outbreaks. That conference dealt with three separate issues: the control of H5/H7 low pathogenic avian influenza in commercial poultry, the control of these viruses in the live bird marketing system, and the use of vaccines to control these viruses. That informal conference resulted in a number of areas of consensus on the three items of discussion, and a few areas of controversy. The informal recommendations of the San Antonio conference were considered formally at the Annual Meeting of the USAHA Committee on Transmissible Diseases of Poultry and Other Avian Species in St. Louis, MO on October 21-22, 2002.

In addition, Resolution 28, concerning the use of avian influenza vaccines to control low pathogenic H5/H7 avian influenza was passed by the Committee at the USAHA meeting in St. Louis, MO, and eventually by the USAHA Executive Board, and received a favorable response from USDA. This policy was implemented in the handling of the low pathogenic H7N2 outbreak in Connecticut in 2003.

Subcommittees were formed at the USAHA meeting in St. Louis, MO to further develop control programs for commercial birds and the live bird marketing systems. Those subcommittees completed the following suggested control plans in late January 2003, and the plans were informally submitted to USDA for use as templates that represented the consensus of opinions of the Committee membership.

A Model Control and Eradication Program submitted to USDA for Consideration in the Development of a National Control and Eradication Program for Low Path H5/H7 Avian Influenza. This Program is based on the following Tenets:

This Model Voluntary Cooperative State – Federal Program to Control and Eradicate Low Path H5/H7 Avian Influenza Infections of Commercial Poultry is State-Based and Coordinated at the Federal Level

Participation in the Program Would Guarantee Federal Assistance Which Would Provide Adequate Indemnity for Fair Market Value of the Birds and Other Costs associated with Containment

I. Commercial Poultry

In order to participate in the Program, States must fulfill three criteria:

1) The participating State must maintain "U. S. H5/ H7 Avian Influenza Monitored State" status under the NPIP program for avian influenza. The proposed NPIP programs have yet to undergo the required approval process to become official programs, and provisions for achieving "U. S. H5/H7 Avian Influenza Monitored State" status need to be promulgated.
2) The participating State must develop a written State diagnostic commercial bird surveillance program, which must be approved by USDA, APHIS, VS.
   a) H5/H7 Avian Influenza should be a disease reportable to the responsible State authority (State veterinarian, etc.) by all licensed or otherwise legally practicing veterinarians in the State. The responsible State authority (State veterinarian, etc.) also should institute an ongoing avian influenza awareness program for all legally practicing veterinarians in the state.
   b) All laboratories that perform diagnostic procedures on avian species (private, State, and university laboratories) should be required to examine all submitted cases of severe, atypical, or otherwise unexplained respiratory disease, gastrointestinal disease, neurological disease, egg production drops, and high mortality for avian influenza by both a USDA approved serological test and a USDA approved influenza virus detection test. All initial cases in which a competent diagnostician would consider avian influenza a reasonable differential should be similarly examined for avian influenza by both serological and antigen detection methods. Memoranda of Understanding or other means should be used to establish testing and reporting criteria and approved testing methods.
   c) All commercial producers should sign Memoranda Of Understanding to support the diagnostic surveillance program by timely submission of appropriate specimens from all flocks with signs or lesions suggestive of avian influenza. Such cases should include severe, atypical, or otherwise unexplained respiratory disease, gastrointestinal disease, neurological disease, egg production drops, or high mortality.
3) The participating State must develop a written State initial containment and control plan, which must be approved by USDA, APHIS, VS.

II. Live Bird Market System

The Cooperative Federal-State Program to Control Low Path H5/H7 Avian Influenza Infections in the Live Bird Market System would be Federally Based and State Assisted.

Participation in the Plan would Guarantee Federal Assistance Including Adequate Indemnity at Fair Market Value of the Birds and for other economic losses.

General:

1. The H5/H7 avian influenza monitoring program for the live bird market system should allow for testing requirements consistent with market history of avian influenza.
2. Cost sharing must be established between USDA and participating States to provide resources for monitoring, compliance, and indemnity.
3. The H5/H7 avian influenza control program that is established for the live bird market system must be mandatory, uniform and linked to federal indemnity.
4. Only States adopting program standards would be eligible for federal indemnity.
5. Education and training will be an important and ongoing requirement. Development of materials supporting H5/H7avian influenza control and biosecurity must continue.
6. USDA must support the dissemination of avian influenza rapid test technology to the regional level and provide some financial support for the ongoing testing at the regional centers.
7. Compliance activity is essential to the success of a program of this nature. USDA must be prepared to augment existing staff to ensure

that the regulatory presence is visible and effective.

Continued Development of a Proposed Plan for the Control of Low Pathogenic H5/H7 Avian Influenza. The USDA, APHIS response to the proposed plan from the USAHA Committee on Transmissible Diseases of Poultry and Other Avian Species was presented and discussed at the USAHA meeting in San Diego, CA on October 13-14, 2003.

Commercial Poultry. The USAHA proposal for low pathogenic avian influenza control in commercial poultry was based on two tenets: (1) the program should be State based and coordinated at the Federal level, and (2) participation in the program would guarantee Federal assistance in the form of indemnification of birds and other costs associated with containment. Regarding the first tenet, the draft USDA, APHIS, VS program for low pathogenic avian influenza surveillance in commercial poultry would be administered by NPIP, and as such would be State based and coordinated at the Federal level. The details of the NPIP proposal were presented and can be seen in the Proceedings of the 107th Annual Meeting of the USAHA that was held October 9-16, 2003 in San Diego, CA or can be viewed on the USAHA website (www.usaha.org). The development of these regulations are moving through the normal process of approval for NPIP and will be considered at the Biennial meeting in San Francisco, CA in June 2004. Regarding the second tenet, current funding does not provide for guaranteed access to Federal indemnity funds. However, emergency funds for indemnification and other costs of disease control can be requested, as was seen in 2002 during the occurrence of low pathogenic avian influenza in Virginia and North Carolina. Furthermore, cooperative efforts between VS and the States are currently underway to develop a National Surveillance System and a National Incident Management System that will address in a broad sense how States and the Federal government will respond cooperatively to significant disease occurrences. This is a major change to the proposed full indemnity proposed by the committee. One of the incentives to states and their poultry industries is that if they complied with the specifics of the plan, then they could be assured that the lack of funding would not delay the onset of a rapid response requiring condemnation and destruction of infected flocks. The lack of that funding in Virginia in 2002 clearly contributed to the spread of the avian influenza and delay of implementing an eradication program. The total costs of the Virginia low pathogenic avian influenza outbreak was greatly increased and the scope of the final eradication program greatly expanded due to this delay. It is so important that state, industry and federal response to an outbreak be early and without delay. "What a difference a day makes."

Live Bird Markets. The USAHA proposal for low pathogenic avian influenza control in live bird markets was also based on two tenets: (1) the program should be Federally based and State assisted, and (2) participation in the program would guarantee Federal assistance in the form of indemnification of birds and other economic losses. Regarding the first tenet, VS has drafted a Uniform Methods and Rules (UM&R) which proposes minimum federal standards for states that wish to conduct a low pathogenic avian influenza control program for live bird market system participants within their State. Details of the USDA, APHIS response were presented and can be seen in the Proceedings of the 107th Annual Meeting of the USAHA that was held October 9-16, 2003 in San Diego, CA or can be viewed on the USAHA website (www.usaha.org). Regarding the second tenet, current funding does not provide for guaranteed access to Federal indemnity funds. However, emergency funds for indemnification and other costs of disease control can be requested, as discussed above. Furthermore, VS did receive some congressionally allocated funds to support the development of the low pathogenic avian influenza programs in commercial and live bird market poultry in the FY 2004 budget.

A new USAHA Transmissible Diseases of Poultry sub committee on the live bird market plan has been appointed and is chaired by Dr. Ernie Zirkle. The proposed plan for the live bird markets was the most contentious. The marketing of live birds and small groups of birds in various types of settings is complex and not conducive to a broad based plan. The well developed plan that the USAHA Transmissible Diseases of Poultry Committee sent to USDA was designed to work in the New York and New Jersey live bird markets with their history of an almost constant state of infection and the potential for being the major source of outbreaks in commercial poultry in the northeast and mid-Atlantic area. One particular issue was the demand for individual bird identification by some and the opposition to that issue because of being considered as unworkable by others. Dr. Zirkle presented his preliminary findings on a research project looking at the feasibility of using individual bird identification and the results can be seen in the Proceedings of the 107th Annual Meeting of the USAHA that was held October 9-16, 2003 in San Diego, CA or can be viewed on the USAHA website (www.usaha.org).

The new live bird market sub committee will now address this and other issues in an attempt to produce a workable plan that could be used in problem markets and alternatives for smaller markets or for markets never known to be a problem with respect to avian

influenza. In any case it is clear that monitoring of these markets needs to be part of a national plan.

DISCUSSION

We need a national plan to control low pathogenic avian influenza infections in commercial poultry in the United States. USDA, APHIS is to be commended for its leadership in this effort and for its inclusion of stake holders in its development. It is not an easy thing to accomplish. International markets are driving the process because of the effects of outbreaks of low pathogenic avian influenza on the economic viability of the poultry industry now so dependent on export markets to make a profit. It is in the best interests of both government and the poultry industry to prevent and control all H5/H7 avian influenza virus infections.

The other major driving force on having a national plan is the consideration of OIE to redefine avian influenza to include not just high path viruses, but all H5/H7 viruses including low pathogenic viruses. The advantage of this change would be to require member countries to report and be held accountable for these outbreaks and thus require eradication with indemnification as it does for high path avian influenza. Our federal government would then be involved from day one in the costs and eradication of an outbreak and full indemnity should be paid. The disadvantage would be that any outbreak of low path infection by H5 or H7 viruses would lead to eradication of many outbreaks that we can now handle by other means.

Recent outbreaks of avian influenza, including low pathogenic avian influenza in Europe may have tempered the rush to change the definition to include all low path H5/H7 viruses. Therefore, we may have some breathing room and more time to think through what should constitute the National Plan to control low pathogenic H5/H7 avian influenza. If the OIE definition does change, then it is my opinion that the federal government required eradication should also provide full indemnity for the poultry industry.

The author recognizes that the work described in this report is a result of a considerable effort by many individuals including the USAHA Transmissible Diseases of Poultry Committee members, numerous USDA, APHIS, NPIP, and poultry industry personnel and particularly recognizes the efforts of Dr. John Smith, Fieldale Farms, as the Vice-Chair of the USAHA Committee on Transmissible Diseases of Poultry and Other Avian Species.

EVALUATION OF IMMUNIZATION OF COMMERCIAL LAYING HENS WITH A KILLED AI VACCINE

Carol Cardona

ABSTRACT

Vaccination may be an important tool in the control of avian influenza (AI); unfortunately, there is little data on its practical application in commercial chickens. Egg laying chickens in California became infected with a low pathogenicity H6N2 avian influenza virus (AIV) in 2000. The virus has persisted on the multi-age production facilities that dominate the California egg industry. In order to eliminate AIV from multi-age egg production facilities, the United States Department of Agriculture and California Department of Food and Agriculture approved the limited use of inactivated, autogenous H6N2 vaccine in California. Uninfected pullets coming onto an infected production facility are considered "at risk" and may, therefore, be vaccinated. This was the first approved use of killed vaccine to control low pathogenicity avian influenza (LPAI) in chickens in the United States and represented an opportunity to study the effectiveness of vaccination in the control and elimination of LPAI from a population of commercial chickens. In order to study the efficacy of vaccination, we have conducted both longitudinal studies following commercial chicken flocks after vaccination and cross-sectional studies on immunity to AI in vaccinated commercial layers. The results of these studies will be discussed.

AVIAN INFLUENZA CONTROL AND MONITORING IN HONG KONG

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SUMMARY

Hong Kong experienced highly pathogenic avian influenza (HPAI) H5N1 outbreaks in 1997, 2001, 2002, and 2003, of which the 1997 breaks caused 18 human influenza cases with six deaths. It then turned avian influenza into a public health issue in Hong Kong. H5N1 HPAI not only occurred in gallinaceous birds on farms and poultry markets, but was also isolated in waterfowls at two waterfowl parks in Dec 2002 outbreak. Following the early 2002 outbreak, Hong Kong government initiated the first recorded one-year vaccination trial in the Far East region, with Nobilis IA inac® (H5N2 inactivated oil emulsion vaccine) in Pak Sha area to control H5N1 HPAI. The trial results showed that infection was not detected in any of the vaccinated flocks under field conditions. In December 2002 H5N1 HPAI outbreaks in two waterfowl parks, outbreaks in five previously unvaccinated chicken farms were followed. Emergency vaccination used in the face of outbreaks on three of the unvaccinated farms, coupled with selective culling and stringent biosecurity, resulted in elimination of H5N1 virus infection from these farms. In these seven years since 1997, substantial and comprehensive control and monitoring systems have been continuously strengthened and enforced with the combination of vaccination as an additional measure to control H5N1 HPAI. A retrospective review of the H5N1 HPAI experience in Hong Kong is discussed in a field perspective in this paper.

INTRODUCTION

Hong Kong experienced highly pathogenic avian influenza (HPAI) H5N1 outbreaks in 1997, 2001, 2002, and 2003. The 1997 outbreaks caused high mortality in chicken farms and 18 human cases with six deaths which led to the depopulation and disinfection of all poultry markets and all chicken farms in the region. 1.3 millions of birds were killed and all imported live chickens banned. In 2001, the outbreaks occurred only in the retail poultry market with no H5N1 infections found on any local chicken farms. As a result, 440,000 birds in retail markets and 800,000 unaffected on-farm older market age chickens were culled. The retail poultry markets were closed and the importation of live chickens banned for several weeks. The outbreaks on farms in early 2002 resulted in culling 900,000 chickens and disruption of poultry trade although no human cases occurred. Until late 2002, high mortality in the outbreaks was only seen in gallinaceous birds on farms (1997, 2002, 2003) and/or in retail markets (1997, 2001, 2002, 2003). In December 2002, outbreaks of H5N1 HPAI occurred in waterfowl (geese, ducks and swan) and other water birds (wild little egrets and captive greater flamingos) at two waterfowl parks in Hong Kong followed by outbreaks on local chicken farms. An investigation team formed to study the 2002 outbreaks recommended further measures to improve farm and market biosecurity (3). Besides the stringent biosecurity and surveillance programs, vaccination was introduced to the region as an additional control measure after a 12-month long vaccination trial conducted between April 2002 and March 2003.

STRENGTHENED BIOSECURITY AND CONTINUOUS EDUCATION

Biosecurity plans have been continuously revised and further enhanced since 1997. The biosecurity plans are based on Hazard Analysis Critical Control Point (HACCP) principles. Biosecurity plans address the major hazards of allowing a disease to enter the population. The control points and possible control measures for the hazard are: movement of infected animals (as carrier animals and infected birds in the incubation phase may appear healthy, avoid purchasing from potentially infected sources, require tests and certification prior to purchase, transport animals in clean vehicles, transport consignments separately), movement of contaminated people, equipment, vehicles (require disinfection and change of clothes before entry to production area), access to contaminated feed or water (request certification, check ingredients, treat water from pond or open tank source), access of wild birds or rodents to farm areas (bird proofing sheds, remove spilt feed), access to pathogen-carrying aerosols (separate farms and sheds by solid barriers or a distance of at least 500 meters), cross-contamination between species (segregate species on farm, in transit and at markets).

The second aspect of the biosecurity plan is to minimize the impact of the disease if it has already entered the population. The control points for minimizing this impact include monitoring the health of the animals and use of rapid diagnosis for the disease (good records are important), isolation of infected and exposed animals, safe disposal of infected carcasses and their waste products, contaminated feed and other risk materials, use of vaccines to increase resistance to the disease, disinfection of equipment and vehicles, choosing impervious construction materials that can be easily cleaned and disinfected, use of batch or all-in all-out systems to break the cycle of infection, restricting movements of animals, people or equipment between animal groups (8).

These are important not only to the farmer but also to all levels of related tradespersons. The most critical factor in determining the success of any biosecurity plan is that all participants must understand their roles in the plan and their significance. Continuous education and introduction of new licensing system help keep the biosecurity

MONITORING AND SURVEILLANCE

Routine monitoring and surveillance is done in the live poultry markets, local farms, other bird collections and wild birds by the Government and the Department of Microbiology, University of Hong Kong. The latter plays an important role in conducting genetic and antigenic characterization of viral isolates. As part of the surveillance program before the introduction of vaccination, blood was collected from pre-sale local and imported poultry to check for exposure to H5 avian influenza virus using standard hemagglutination inhibition (HI) test (1) for specific antibody levels. If necessary, cloacal swabs were collected for rapid diagnosis for viral antigens using Directigen and/or viral genomes using real-time RT-PCR before being subjected to virus isolation for final confirmation.

USE OF VACCINE AS AN ADDITONAL CONTROL MEASURES

Vaccines have been used in other countries to assist in the control of avian influenza. Countries using vaccines against AI viruses include Italy (2), USA (6), Mexico (10) and Pakistan (7). Mostly vaccination has been directed against low pathogenic avian influenza (LPAI) but Mexico and Pakistan have successfully used vaccine against H5 or H7 HPAI (5). Following the February-April 2002 outbreak, the Hong Kong government initiated an one-year long vaccination trial using Nobilis IA inac® (H5N2 inactivated oil emulsion vaccine) in 22 chicken farms in Pak Sha area to control H5N1 HPAI. This was the very first well-designed vaccine trial for HPAI control in the Far East Asia. Monitoring flocks for the presence of avian influenza depended on clinical monitoring, serological and virological testing of sentinel birds and sick/dead birds (if any). As the neuraminidase antigens (N2) in the vaccine differ from the endemic strain (N1) in Hong Kong, differentiation between vaccination and natural challenge in chickens showing positive antibody titers was possible at that time only by testing neuraminidase antigen - "Differentiating Infected from Vaccinated Animals (DIVA)" principle. However, this differentiation method can be interfered as H9N2 and H6N1 avian influenza viruses can also circulate in poultry in Hong Kong.

In early April 2002, in the first round of vaccine trial, all on-farm chickens between 8-55 days of age were vaccinated and boosted four weeks later. Subsequently, all new batches of chicks were vaccinated at 8-10 days of age and boosted after four weeks. Thirty individually identified unvaccinated chickens serving as sentinel birds were placed in each batch. During the trial period, for sale purpose, blood was collected and tested for antibody levels (HI titers) from 30 sentinels and 30 vaccinated chickens at four weeks after the first and second vaccination. All sentinels or vaccinated birds that became sick or were killed by any unknown cause(s) were subjected to necropsy investigation and tested for avian influenza virus with standard laboratory methods (1). Prior to sale, 60 samples per batch had cloacal swabs collected and tested for the possible presence of H5 virus by NASBA (4) or real-time RT-PCR (9) in addition to the serological test.

The trial results showed that infection was not detected in any of the vaccinated flocks under the field condition. In response to H5N1 HPAI outbreaks in two waterfowl parks and in wild water birds in Hong Kong in December 2002, the vaccination program was extended to 53 farms in high risk areas. Encouragingly, HPAI infection was not detected in any of the vaccinated flocks in the Pak Sha area during or after these outbreaks.

Between late December 2002 and January 2003, outbreaks in five unvaccinated chicken farms occurred. Immediate quarantine and movement control were put in place while two involved farms were completely depopulated. Emergency vaccination was used on the remaining three unvaccinated farms which formed part of the last phase of the vaccine trial. Together with selective culling, and stringent biosecurity, the vaccination helped control and eliminate H5N1 virus infection from these farms. This was demonstrated by field evidence from the daily monitoring of other non-infected sheds within the farms and the surrounding farms and laboratory results from virus culture using

swabs from birds and cages from these affected farms. Following the vaccination trial, universal vaccination of local chicken farms and Mainland farms supplying chickens to Hong Kong was introduced.

CONCLUSION AND DISCUSSION

These programs will be closely monitored and may need to be revised in order to any new threats that may be identified. Implementation of such a comprehensive control and monitoring program as the one presented above, has only been possibly through the support of the industry and great cooperation between legislators, regulators, farmers and various public service personnel (3, 8). Without these measures in place, any kinds of vaccination could be undermined by influences and contaminants beyond producer's control. The net result is that the people of Hong Kong now have a greatly reduced risk of exposure to avian influenza and the poultry industry has greater security with respect to future production.

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(The details of the vaccination trial will be published as a full-length paper in the near future.)

INCREASED RESISTANCE OF VACCINATED TURKEYS TO EXPERIMENTAL INFECTION WITH AN H7N3 LOW PATHOGENICITY AVIAN INFLUENZA VIRUS

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INTRODUCTION

Avian influenza viruses may be classified on the basis of the severity of the clinical signs they cause in susceptible birds. Low pathogenicity avian influenza (LPAI) may be caused by viruses belonging to all 15 hemagglutinin subtypes (H1-H15) and produces a mild disease in susceptible poultry. Highly pathogenic avian influenza (HPAI) is, in contrast, a systemic viral disease of poultry with mortality that approaches 100% in many gallinaceous birds (1). This form of the disease is caused only by certain strains of H5 and H7 viruses that contain multiple basic amino acids at the cleavage site of the hemagglutinin molecule. Turkeys have been shown to be highly susceptible to infection with both HPAI and LPAI viruses. They also develop a more severe clinical condition following infection with LPAI (6, 9). Field observations suggest that they are more susceptible and may act as amplifiers of infection (1).

Guidelines for the control of HPAI are contained in EU Directive 92/40/EEC (4). In the past, vaccination against HPAI or LPAI of H5 and H7 subtypes has not been considered a possible option for their control, and restriction measures and stamping out are currently the major tools for the control of AI. However, vaccination is attractive as an aid to control infection because it does appear to reduce the excretion of virus from birds challenged subsequently (2) and this may reduce both the environmental virus load for HPAI viruses and the likelihood of mutation to virulence for LPAI H5 and H7 viruses. No data are available on whether vaccinated birds are less susceptible to infection. If this was the case, the coupled effect of reducing the viral load in the environment and the reduction of susceptibility to field challenge would represent valid reasons to implement vaccination programs during eradication campaigns, particularly in densely populated poultry areas (DPPA).

Conventional inactivated oil emulsion vaccines prepared with the homologous strain work well but do not enable the serological detection of field exposure unless identifiable unvaccinated sentinels are left in the flock. Similarly inactivated vaccines containing an AI strain which has the same H subtype as the field virus but a different N enable field exposure to be identified through the application of a serological test able to detect the specific anti-N antibodies of the field virus. This system allows the "DIVA"- (differentiating infected from vaccinated animals) strategy to be used (3).

The aim of the present study was to establish the degree of susceptibility and virus shedding in turkeys vaccinated with an influenza strain containing a heterologous neuraminidase to that of the challenge virus.

MATERIALS AND METHODS

Sixty commercial turkeys, hatched in isolation, were divided randomly in two groups of 30. One group was vaccinated with a commercially available inactivated oil emulsion product containing the strain A/ty/Italy/99/(H7N1). The remaining group was left as unvaccinated controls. Each group was subsequently divided into 3 groups of 10 and challenged with different dilutions (10^2, 10^4, 10^6 EID_{50}/0.1ml) of a LPAI isolate A/ty/Italy/8000/02(H7N3) obtained during the 2002-2003 Italian epidemic. Infected birds were observed daily with tracheal and cloacal swabs collected at regular intervals for antigen detection by a commercially available antigen-capture enzyme immuno-assay (AC-EIA), virus isolation and real-time RT-PCR (RRT-PCR). Pre- and post-infection serology was also performed by means of AGP and HI test.

RESULTS

No clinical signs were observed in any of the birds belonging to the vaccinated groups. Clinical signs were observed in the unvaccinated birds infected with 10^4or 10^6 EID_{50}/100µl. 5/10 birds challenged with 10^4 EID_{50} showed depression associated with a mild diarrhoea and respiratory signs starting on day 4 post-infection. Three birds challenged with 10^6 EID_{50} also exhibited sinusitis, characterized by swelling of the infraorbital sinuses. All clinical signs, except for the

sinusitis, were self-limiting and disappeared by day 20 post-infection.

The results of virological investigations, RRT-PCR and AC-EIA on cloacal and tracheal swabs, and the serological results indicated that infection was not achieved in the birds challenged with 10^2 EID_{50}/100µl and in the vaccinated birds challenged with 10^4 EID_{50}/100µl. On the contrary, infection was achieved in the naïve birds challenged with 10^4 EID_{50} as well as in the birds challenged with 10^6 EID_{50}, regardless of their state of vaccination. A reduction of the number of positive samples and of the duration of shedding in the vaccinated compared to the unvaccinated controls was detected by all three tests (virus isolation, RRT-PCR and AC-EIA) in the samples obtained from pooled tracheal swabs. A similar result was obtained from cloacal swabs processed for attempted virus isolation. However, the latter samples were positive using the RRT-PCR test both in the vaccinated and in the unvaccinated populations up to the termination of the experiment (day 20 post challenge). Overall the number of samples positive for viral RNA was greater in the unvaccinated group than in the vaccinated group. The current experiment did not determine if the amounts of virus detected by the RRT-PCR were infectious for birds.

For the calculation of the viral titer necessary to infect the vaccinated vs. the unvaccinated birds, a total of 4/30 birds resulted infected among the vaccinated birds and 15/30 birds resulted infected in the unvaccinated group. The application of the Spearmann Karber formula indicated that a dose of $10^{6.2(\pm 0.16)}$ was required to infect the vaccinated group while the unvaccinated group required $10^{4(\pm 0.17)}$.

The application of the Fisher test indicates that these values differ significantly (*p* 0.016). Thus, a statistically significant higher dose is required to infect vaccinated birds compared to naïve birds.

CONCLUSIONS

The results of the experiment indicated that infection was achieved in naïve birds with 10^4 EID_{50}, while vaccinated birds were resistant at this challenge dose. Vaccinated and unvaccinated birds were susceptible to infection with 10^6 EID_{50} although the duration and/or the number of birds shedding was reduced in the vaccinated group.

A predictable discrepancy was observed when comparing the results obtained for the AC-EIA, the RRT-PCR, and attempted virus isolation in eggs. Considering virus isolation as the "gold standard", the AC-EIA appeared to be less sensitive and RRT-PCR more sensitive. The reasons for this probably lie in the fact that the kit was not developed for poultry but for humans and is not validated in avian species. In previous studies the same AC-EIA was applied on samples of avian origin and a sensitivity of 79 % relative to virus isolation was obtained (5). The RRT-PCR is probably able to detect non-viable virus, or amounts smaller than one infectious dose (7).

The data presented indicate that heterologous vaccination in the framework of a "DIVA" strategy may be appropriate as a tool to support eradication measures employed during an AI outbreak, particularly in areas with high densities of susceptible animals. In addition to the well-known effect on reduction of shedding of infectious virus (8) vaccination generates a higher resistance to infection. The combination of these two effects is particularly useful in areas at risk as in DPPA. However, vaccination alone will not achieve the goal of eradication. Strict biosecurity measures and restriction policies represent the main tools to prevent the introduction and perpetuation of avian influenza infections in domestic poultry. Vaccination should be only considered as a tool to maximize the effect of sanitary measures in the face of an outbreak or when the risk of introduction in DPPA exists.

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IMMUNOGENICITY OF A FOWLPOX VECTORED AI VACCINE: EFFECT OF ADJUVANT AND MULTIPLE IMMUNIZATION IN SEROCONVERSION AND IN PROTECTION VS CHALLENGE WITH HP AVIAN INFLUENZA IN BIRDS WITH PRE-EXISTING FOWLPOX IMMUNITY

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ABSTRACT

Avian influenza-Fowlpox vaccine, live fowlpox vector, H5 subtype (vFP89, licensed product Merial Select, Inc., Gainesville, GA), has been shown to be efficacious against the highly pathogenic (HP) Avian influenza (AI) virus A/chicken/Queretaro/15588-19/95 (H5N2), and eight other different HP H5 AI viruses (1, 2). However, the use of this vaccine in birds with pre-existing immunity to fowlpox has provided partial protection only against this HP AI challenge (3). The initial objective of this study (experiment 1) was to evaluate the primary and booster humoral immune responses to Avian influenza-Fowlpox, H5 vaccine (vFP89), administered to SPF chickens previously vaccinated with fowlpox (FP) using different doses and formulation (adjuvant administration). The second objective (experiment 2) was to confirm that the formulation of the vaccine in combination with the adjuvant induced immunity in SPF birds previously vaccinated with FP in a vaccination-challenge trial.

Results from the first experiment showed that birds vaccinated at one-day of age with FP, and vaccinated with the vFP89-adjuvant formulation at 21 and 42-days of age, seroconverted as measured by the hemagglutination-inhibition (HI) test using the homologous vaccine antigen A/Turkey/Ireland /1378/83 (H5N8). Out of 15 vaccinated birds, 11 seroconverted (HI GMT 15) 21 days after the vFP89-adjuvant vaccination at 21-days of age, and 14 seroconverted (HI GMT 45) 21 days after the vFP89-adjuvant booster vaccination at 42-days of age. A similar vaccination schedule without the adjuvant formulation only induced seroconversion (HI GMT 11) in nine out of 14 birds, 21 days after the boost at 42-days of age with the vFP89 vaccine. When the dose of the vFP89 vaccine was increased ten fold (10X) at the vaccinations at 21 and 42-days of age, seroconversion occurred in 13 of 15 vaccinated birds (HI GMT 26) 21 days after the vFP89 booster vaccination at 42-days of age. In contrast, birds that were initially vaccinated with vFP89 at one or 21-days of age, and booster vaccinated with vFP89 at 21 or 42-days of age, had 100% seroconversion. The HI GMT's of these birds had a range of 34 to 160 at 21 to 63 days post-vaccination. The HI test was also run using the AI A/Turkey/Wisconsin/68 (H5N9) antigen. However, the HI GMT's obtained with the heterologous antigen were <8.

Results from the second experiment, the vaccination-challenge trial, indicated 82% of the birds vaccinated at one-day of age with FP, and vaccinated with the vFP89-adjuvant formulation at 21 and 42-days of age, were protected against the challenge with the HP AI virus A/chicken/Queretaro/15588-19/95 (H5N2) as shown by morbidity and mortality. Birds vaccinated with the same vaccination schedule without the adjuvant formulation showed no adequate protection against morbidity (36%) and mortality (55%). Single vaccination with the vFP89 vaccine, with or without the adjuvant formulation, at either 21 or 42-days of age, did not protect birds from morbidity (9-36%) or mortality (18-36%). In birds without pre-existing immunity to FP, a single application of the vFP89 vaccine at 21-days-of age, alone or in combination with the adjuvant formulation, induced protection to morbidity and mortality. The protection conferred by the vFP89 vaccine without the adjuvant was 100% and 80% for the vaccine-adjuvant formulation.

HI serology tests were conducted as described in the first experiment. Results were pending at the time the abstract was submitted.

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AVIAN INFLUENZA H6N2 IN CALIFORNIA BROILER CHICKENS AND TURKEYS-2002

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ABSTRACT

Avian influenza (AI) is a viral disease of various species of birds including domestic poultry caused by Orthomyxovirus (2). The clinical signs of AI in domestic poultry can range from an asymptomatic infection to respiratory signs, decreased egg production to a severe systemic infection with high mortality (2). The severity of the clinical signs and pathology due to AI virus depend on the pathogenicity of the virus, whether it is a low or high pathogenic strain (such as H5 and H6), the presence of concurrent infections, age and species of birds and nutritional and environmental factors (2). AI outbreaks between 2000 and 2002 due to the low pathogenic virus, H6N2, have been reported in layer type chickens in California (1, 3). This paper describes the occurrence of AI H6N2 in broiler chickens and turkeys in California during 2002.

A total of 50 live and seven dead broiler chickens ranging in age from 41 to 60 days from six different ranches were submitted to the laboratories with history of respiratory signs, increased mortality and high condemnation rates at processing plants. Gross lesions in most of these birds included increased mucus in the trachea and in some cases fibrinous exudate in the air sacs and bronchi. Microscopically, most of the birds had mild to severe diciliation with lymphoplasmacytic inflammation in the sinuses, turbinates and tracheas. There was a similar inflammation in the bronchi and also in the interstitium of the lung. In cases complicated with *Escherichia coli*, there was severe fibrinosuppurative inflammation of the air sac, pericardium, pleura and lungs. Sera from most of the birds were positive for AI by AGID and ELISA. Avian influenza virus was isolated from the trachea and lung and cecal tonsils.

In addition to the broiler chickens, 12 live turkeys ranging in age from nine weeks to 13 weeks, from three different ranches, were presented with a history of respiratory signs. In addition, one ranch experienced a severe increase in mortality. Most of the turkeys had swollen sinuses with mucoid exudate and similar exudate in the trachea. Sera from most of these birds were also positive for AI both by AGID and ELISA. Avian Influenza virus was isolated from the trachea of most birds and from the cecal tonsils from the one group in which isolation was attempted.

The AI virus isolated from both broiler chickens and turkeys was determined to be H6N2 at NVSL (D. Senne, Ames, IA). The virus was determined to be of low pathogenicity to chickens by pathogenicity studies (NVSL, Ames, IA). Immunohistochemistry was performed (Dr. D. Swayne, USDA-ARS, Southeast Poultry Research Laboratory, Athens, GA) on paraffin-embedded blocks containing trachea, air sac and lungs from some of the cases (both broiler chickens and turkeys), which revealed scattered AI nucleoprotein in the cytoplasm of the epithelial cells of the trachea and bronchi.

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SURVIVAL OF EXOTIC NEWCASTLE DISEASE VIRUS IN COMMERCIAL POULTRY ENVIRONMENT FOLLOWING REMOVAL OF INFECTED CHICKENS

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During the first weeks of 2003 after Exotic Newcastle Disease (END) was confirmed in commercial layer flocks in Southern California, it became apparent that the virus survival information in the literature varied widely and was difficult to extrapolate to current local conditions. The END Task Force used the information available in the literature and the recommendations of research scientists to establish protocols for safely handling manure from infected and depopulated premises.

In an attempt to gain more directly applicable data, this virus survival study was designed and implemented. Two of the first END infected layer ranches were selected for environmental drag swab sampling. With the cooperation of the owners, the environmental swab sampling was conducted in several types of houses immediately after the removal of the END-infected chickens to determine the survival time of the END virus in the poultry houses and in the manure on these ranches.

A total of 250 pooled swab samples were analyzed. Fifteen pools were positive for END virus. Forty percent of the pooled samples were positive at the start of sampling immediately following depopulation. Last positive pooled sample was at 16 days post depopulation. No END virus was isolated after the 16th day after depopulation from any of the samples.

VACCINE PROTECTION IN CHICKENS AND TURKEYS AGAINST THE 2002 EXOTIC NEWCASTLE DISEASE VIRUS

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Newcastle disease vaccination is widely practiced in the USA with the majority of commercial broiler breeders, layers, and turkeys receiving multiple vaccinations during their lifetime. Initial vaccination is with a live, low-virulence lentogenic virus followed by either repeated live lentogenic or inactivated vaccine. The objectives of the present study were to extend the knowledge of protection against U.S. exotic Newcastle disease (END) virus by live and inactivated Newcastle disease virus (NDV) B1 vaccines, and determine immunity of SPF and commercial chickens and turkeys following lethal challenge with a California 2002 (CA02) END virus isolate.

Initial experimentation was designed to assess protection of SPF chickens receiving a single dose of a commercially available inactivated or live NDV B1 vaccine from CA02 challenge, as well as different doses of live vaccine followed by challenge. In a subsequent experiment, field NDV vaccinated commercial broiler-breeders and broilers in Georgia

were challenged with CA02. The results indicated both live and inactivated vaccines protected SPF chickens from morbidity and mortality due to a lethal CA02 challenge. However, the vaccines were unable to prevent infection and challenge virus shed as determined by virus isolation from oral and cloacal swabs. In contrast, commercial broilers were susceptible to CA02 challenge, in spite of receiving two live virus vaccinations in the field at 1 and 17 days-of-age. Seventy-five percent of these birds succumbed to challenge. Commercial broiler-breeders were resistant to morbidity and mortality from challenge and CA02 virus was recovered from ≤ 30 % of challenged birds.

Preliminary studies in turkeys indicated that 21-day-of-age SPF birds were susceptible to END challenge. In contrast, 40-day-of-age NDV-antibody-negative commercial turkeys appeared resistant to END challenge and did not exhibit overt disease. For vaccine-challenge studies, the presence of maternal antibody in 10-day-of-age commercial birds at vaccination made protective immunity difficult to assess following challenge at 24-days-of-age. A positive correlation was observed between pre-challenge antibody titers and protection.

In conclusion, the results demonstrated that current NDV vaccines can protect against lethal END challenge among chickens and turkeys. In contrast to "normal" NDV vaccine strategies, the timing and protocol of vaccination for commercial birds with maternal antibody should be evaluated during an END outbreak.

THE ROLE OF VACCINATION IN THE ERADICATION OF VIRULENT NEWCASTLE DISEASE IN AUSTRALIA PARTICULARLY IN RELATION TO THE EGG INDUSTRY

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SUMMARY

Australia claimed freedom from Newcastle disease (ND) in 2001 following the successful eradication of virulent ND from infected farms. The virulent virus was shown to have been derived from a series of mutational changes of the F protein cleavage site in endemic lentogenic virus, with a pronounced increase in virulence from the precursor virus. However, the detection of further precursor ND viruses in 2001 and outbreaks of virulent ND in NSW and in Victoria in 2002 again associated with mutated endemic viruses, prompted the formation of a ND National Management Group and a Steering Committee. Those committees developed a ND Management Plan. An integrated risk management approach was taken involving five operational projects. A control project involving biosecurity plans, strategic vaccination together with a national surveillance project to detect the presence of precursor and virulent ND virus (vNDV) were the major components of the Plan. Because of the potential for vNDV re-emergence, a vaccination strategy was developed that aimed to out-compete precursor viruses that have sequences close to that of the sequences of vNDV. The only live vaccine permitted is the lentogenic V4 strain. Vaccination uptake in states where vaccination is compulsory has been high although there has been some resistance amongst smaller layer farmers due to the cost of inactivated vaccine. The extent to which vaccination can prevent the spread of precursor virus seems paramount to the success of the program. Failure to communicate with the fringe sector of the industry on the need to vaccinate and to uphold high levels of biosecurity could allow precursor viruses to persist and prevent total eradication.

INTRODUCTION

The occurrence of virulent ND in Australia due to the mutation of previously endemic lentogenic ND viruses was reviewed for the 48th meeting of this conference (5). Further outbreaks of virulent ND occurred in two poultry production areas in NSW in 2000. A national serological and virological survey in 2000 failed to identify any precursor or virulent viruses. Following slaughter of poultry on the infected farms, Australia was declared free of ND in 2001. However, one isolated outbreak in Victoria in 2002, followed by three further outbreaks in NSW later that year, prompted the introduction of ND vaccination with the V4 strain of all commercial chicken farms in the Sydney area. These outbreaks also prompted the National Management Group (NMG), a high level government-industry committee responsible for emergency response plans, to request the development

a National ND Management Plan. The Steering Committee of the NDNMG recommended an integrated risk-managed approach. This paper provides an outline of the operational projects created to meet the set objectives of the program with particular emphasis on ND vaccination and the egg industry.

NATIONAL ND MANAGEMENT PLAN

The integrated risk management approach was aimed to deliver the following goals:

1. Minimize the risk of ND outbreaks from Australian-origin virulent viruses
2. Protect the status of non-infected flocks and regions; and
3. Reduce the social, economic, and trade impact of ND at farm, regional, and national levels.

The operational projects to deliver these goals comprised:

1. A control project involving biosecurity plans, strategic vaccination, and other agreed standard operating procedures (SOP).
2. A national surveillance project to detect the presence of precursor and virulent ND viruses.
3. A communication project to promote awareness in the poultry industry.
4. A research and development project to support the Plan.
5. A management and evaluation project to provide co-ordination and review implementation of the Plan.

LEGISLATIVE BACKING TO THE PLAN

The commonwealth and state governments have enacted legislation in the past to support exotic disease control in their respective jurisdictions. This enables regulations to be adopted in relation to compulsory vaccination. By early 2004, it is anticipated that five of the six states will have made ND vaccination compulsory.

VIRULENT ND IN THE LAYER INDUSTRY

Although the majority of outbreaks have occurred on broiler farms, 21 of those 27 cases in broiler chickens probably arose through transmission from nearby broiler farms. One outbreak occurred in an isolated broiler breeder flock. Ten of 13 ND outbreaks on layer farms occurred in isolated flocks without clear epidemiological connection to each other. On each occasion, the outbreak was associated with the introduction of naive pullets (NDV antibody negative) onto a multi-aged layer farm. Therefore, it was considered that virulent ND precursor virus, persistent in ND antibody positive older birds on these sites, mutated during propagation through the newly introduced pullets, resulting in a clinical outbreak.

RISK-BASED VACCINATION OF THE LAYER INDUSTRY

Because of the potential for vNDV re-emergence, a vaccination strategy was developed that aimed to out-compete precursor viruses that have a fusion protein gene sequence similar to that of the virulent virus. A risk-based approach was developed that allowed states or areas to claim an exemption from compulsory vaccination based upon surveillance data. Only one State, Western Australia made a successful case to avoid compulsory vaccination but will undergo intensive surveillance. A risk management approach requires all other commercial flocks in Australia to be vaccinated according to prescribed SOPs.

The only live vaccine permitted is the lentogenic V4 strain (6). Whilst V4 vaccine has some inherent limitations in relation to efficacy in the face of NDV maternal antibodies (9) and in layer flocks in cages (1), the proposed surveillance program involving auditing for compliance and serological monitoring will permit necessary amendments to the SOPs. Minimum serological hemagglutination inhibition (HI) titers were set at $\geq 2^3$ in rearing, and $\geq 2^5$ in production, following a single live vaccination in floor-reared flocks, or two live vaccinations for cage-reared flocks, followed by an inactivated vaccine at 12 –18 weeks of age.

Currently (2003), only one live V4 vaccine is available for use in Australia. Two other Australian vaccine manufacturers are trialing live V4 vaccines prior to making application for registration. One of those vaccines has undergone back-passage in chickens to attempt to improve infectivity (2). A recent report by Underwood and De Laney – unpublished) indicated that the Bioproperties Vaxsafe ND V4 Vaccine was able to produce comparable levels of protective antibody to that of the currently registered vaccine.

BIOSECURITY AND QUALITY ASSURANCE IN THE LAYER INDUSTRY

The Australian egg industry has undergone considerable contraction in ownership over the past five years with over 80% of production now under the control of three poultry companies. The total number of egg producing farms is estimated to be approximately 900; however, only about 270 of these contain more than 10,000 hens. This intensification of the industry has allowed the development of improved biosecurity programs (4) and the extension of this into a Quality Assurance Program incorporating food safety,

biosecurity, welfare, and product labelling. However, a considerable number of smaller producers remain refractory to biosecurity programs and continue to pose a risk to larger producers. Therefore, the ND control strategy incorporates monitoring of biosecurity programs. To increase incentives to implement biosecurity, an attempt is being made to link compensation for the cost of eradication to the level of biosecurity procedures adopted by the farmer.

FUTURE PROSPECTS OF ERADICATION

Australia would appear to have experienced a rather unique event in terms of the mutation of vNDV from an endemic lentogenic strain (7). The factors that encourage precursor and vNDVs to emerge are not well understood, although flock immunosuppression due to concurrent MDV, IBDV or CAV infection has been suggested to alter selection pressure during virus propagation through the flock, assisting evolution to virulence. Alternatively, Westbury (8) has suggested that vNDV may have emerged slowly in accordance with the quasi-species concept (3) with evolutionary selection pressures on a heterogeneous population of NDVs causing those sub-populations to emerge that are best adapted to the changing poultry growing environment.

Knowledge of the epidemiology of the precursor viruses and the capacity of live V4 vaccination to eliminate the precursor viruses is not well understood. A research project on the latter aspect has been initiated at the Australian Animal Health Laboratory (AAHL). Some evidence that the initial use of live V4 vaccine in one broiler growing area in NSW resulted in no re-occurrence of vNDV suggested that live V4 vaccine was beneficial. A nationwide survey conducted in 2000 found no evidence of precursor viruses on farms where V4 vaccination was undertaken. However, a further outbreak of vND and the detection of precursor viruses in 2002 on non-survey farms, indicated that unvaccinated poultry farms were still at risk. Whether vNDV did spread between distant poultry growing areas in Australia is not known. However, it is known that precursor viruses existed in each of the outbreak areas and therefore had the potential to mutate into vNDV.

In those states where vaccination has become compulsory, there has been a high uptake of the live vaccine, however there remains resistance to the use of inactivated vaccine, particularly on small layer farm units. There also remains concern that the interference of vaccine replication by ND maternal antibody in broiler flocks, and the poor response to vaccination of layer pullets reared in cages, which may allow continued circulation of precursor virus in broiler and layer flocks, ultimately leading to further outbreaks of vND. A serological surveillance program has been established to monitor the response to vaccination in the layer industry to determine the current status of these flocks.

Whether the risk-based strategy proposed by the industry-government NDMG will succeed will depend upon a number of factors, some of which are related to the biology of the virus, and others dependent upon the enthusiasm with which the poultry industry and government face the problem. The extent to which vaccination can prevent the spread of precursor virus seems paramount to the success of the program. Failure to communicate with the fringe sector of the industry on the need to vaccinate and maintain high levels of biosecurity could allow precursor virus to persist and prevent total eradication.

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THE MOLECULAR BASIS OF THE POULTRY COCCIDIOSIS VACCINE, COXABIC®

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SUMMARY

CoxAbic® is a subunit vaccine that is based on the use of purified, sexual stage, gametocyte antigens from *Eimeria maxima*, to immunize breeding hens just prior to the start of lay, which via maternal immunity, provide protection to broiler chicks against coccidiosis. Using a histological, proteomic, biochemical and molecular biological approach, the molecule components of CoxAbic® were characterized to improve our understanding of the mechanism of action of this vaccine. It was found that the gametocyte antigens in CoxAbic® are precursor proteins found in the wall forming bodies of macrogametes, which are proteolytically processed during parasite development to smaller tyrosine-rich fragments that are then enzymatically cross-linked to form the hardened barrier of the oocyst wall. These results suggested that CoxAbic® interferes with oocyst wall formation in the parasite.

INTRODUCTION

Coccidiosis is an extremely important disease of chickens worldwide. It results in estimated losses to the broiler industry alone of more than US$1 billion per year. Coccidiosis is caused by infection with any of seven species of the apicomplexan protozoa, *Eimeria*, including *Eimeria acervulina, Eimeria brunetti, Eimeria maxima, Eimeria mitis, Eimeria necatrix, Eimeria praecox*, and *Eimeria tenella*. Of these, *E. tenella, E. maxima*, and *E. acervulina* are considered the most problematic. Symptoms of coccidiosis include listlessness, anemia, watery or bloody diarrhea (depending on the infecting species), weight loss and poor feed conversion ratios. The disease can be fatal to heavily infected chickens. Currently, coccidiosis is mainly controlled by the use of in-feed medication but the increasing incidence of drug resistance by the parasite (7) coupled with increasing consumer demand for decreased use of drugs in agricultural management schemes has encouraged the development of a subunit vaccine against coccidiosis, CoxAbic®.

COXABIC®

CoxAbic® is a novel vaccine produced by ABIC Ltd., Israel, based on using three major affinity purified, native antigens (of 56kDa, 82kDa and 230kDa) isolated from the macrogametocyte (female sexual) stage of development of *Eimeria maxima* to vaccinate laying hens just prior to the start of their laying period (8). Protective maternal antibodies are transferred via the egg yolk to offspring chicks, which hatch with high titers of maternal antibody. These maternal antibodies act to reduce oocyst shedding for the first 2-3 weeks of the chickens' growth period. This, in turn, leads to a 60-80% lowering of the peak litter oocyst counts, which usually occurs at 3-5 weeks of age (8).

A multicentered, multinational series of field trials were carried out in four countries from four different continents (Argentina, South Africa, Thailand and Israel). Broiler chickens from CoxAbic® vaccinated hens that were raised without anticoccidial drugs in their feed performed as well and, often, even better than control broiler chicks given feed containing coccidiostats (8). This improved performance was particularly apparent in terms of feed conversion ratio, which was found to be significantly better in CoxAbic® chicks as compared to drug treated controls.

Thus far, performance results from several million broiler chickens vaccinated with CoxAbic® and raised without coccidiostats have been excellent (6). The surprising finding that the performance data on CoxAbic® vaccinated chickens even surpassed that of broilers raised with coccidiostats in their feed, is probably due to the toxic side effects of some of these compounds. Work is now in progress to further scale up the production, registration and marketing of CoxAbic®. Simultaneously, studies are also being performed (see below) to further our understanding of the mode of action of this vaccine and for production of the recombinant version.

OOCYST WALL FORMATION

Eimeria maxima, like every other apicomplexan, has a complex lifecycle that includes asexual (sporozoites and merozoites) and sexual (macrogamete and microgamete) reproductive stages. The oocyst wall of *E. maxima* is formed from specialized organelles called "wall forming bodies", found exclusively in the macrogametes. The antigenic profile of the macrogamete is dominated by two proteins, one of 56 kDa (gam56) and one of 82 kDa (gam82), two of the principle components of CoxAbic®. A biochemical characterization of these proteins revealed that they are glycosylated, acidic proteins, and do not form homo- or hetero-dimers (1). The genes encoding these proteins were isolated using the polymerase chain reaction and oligonucleotide primers designed from the amino terminus of the proteins, and tryptic peptide fragments from cDNA synthesized from gametocyte mRNA (2,3). The genes were sequenced and, although they do not show any homology to any previously identified gene in any databases, they do share one feature in common with each other and other oocyst wall proteins that had been partially characterized; they were all rich in tyrosine, an amino acid that has been implicated in the stabilization of extracellular matrices in a number of organisms, widely distributed in nature, such as yeast, insects, worms and sea urchins. Further characterization of the genes encoding gam56 and gam82 showed that they are present in the genome as single-copy genes that are developmentally regulated (2,3).

Molecular tools, such as antibodies, were raised to genetically engineered versions of gam56 and gam82 to use in a study to determine where the two proteins were found within the parasite. The antibodies localize to the wall forming bodies and also to the oocyst wall of the developing parasite (5). These localization studies, together with the characterization of the genes encoding gam56 and gam82, suggest that the oocyst wall of *Eimeria* is composed of tyrosine-rich proteins that are derived from wall forming bodies in the macrogamete of the parasite.

Further molecular studies revealed that gam56 and gam82 represent large precursor proteins that are broken down during oocyst development to smaller tyrosine-rich fragments, falling into two clusters of 8-12 kDa and ~30 kDa (3,4). These observations indicate that the first step of oocyst wall formation, after the synthesis of the precursor proteins, is the conversion of these proteins, probably by a protease, to a size that would be more suitable for incorporation into the developing wall. The question is, how are these proteins incorporated and hardened to form the oocyst wall?

The role of tyrosine in the stabilization of extracellular matrices has been investigated in a number of organisms, and it is thought that the formation of protein-dityrosine crosslinks leads to dehydration and hardening of proteins. The presence of dityrosine can be detected by its natural blue autofluorescence under UV light. When *E. maxima* oocysts were visualized microscopically under UV light, they fluoresced vividly, indicating the presence of dityrosine-protein crosslinks (4). In fact, dityrosine, as well as DOPA, another product of tyrosine oxidation implicated in the synthesis of structural materials, was also able to be measured biochemically in oocyst extracts and discovered to be present at levels much higher than usually seen in a wide range of organisms or disease states (4). These findings suggest that the next step in oocyst wall formation, after fragmentation, is the formation of dityrosine-, and possibly DOPA-protein crosslinks. But how is this done?

Oxidative enzymes such as peroxidases have been implicated in protein-dityrosine crosslinking, therefore a novel approach was taken to identify whether macrogametes possessed peroxidase activity and, if so, where. Host tissues infected with *E. maxima* were incubated with a specific substrate for peroxidase. Enzyme-mediated catalysis of this substrate stains the active sites black, which are easily identifiable by light microscopy. This experiment revealed peroxidase activity only in the wall forming bodies of the macrogametes and the oocyst walls of the parasite (4).

CONCLUSION

In conclusion, this research has provided evidence to support the hypothesis that pre-existing precursor proteins, such as gam56 and gam82, that are found in the wall forming bodies of macrogametes of the apicomplexan parasite, *E. maxima*, are initially fragmented in an enzyme-driven process, then oxidized in a peroxidase-mediated event, to give rise to dityrosine- and possibly DOPA- protein crosslinks, which lead to the hardening of the oocyst wall. These results have shown that the parasite has developed an efficient, fast and resourceful mechanism to form the oocyst wall, decreasing its vulnerability to adversity, which it is constantly exposed to throughout its lifecycle. However, this process is also a potential Achilles' heal for the parasite because it is fundamentally crucial for the parasite's successful transmission and, therefore, survival. Thus, interference with oocyst wall formation, as appears to occur following maternal immunization with CoxAbic®, is an exciting and novel control strategy for coccidiosis in broilers.

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COCCIDIOSIS IMMUNITY DEVELOPMENT AFTER *IN OVO* DELIVERY OF *EIMERIA* PARASITIC STAGES

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INTRODUCTION

When *E. tenella* oocysts, sporocysts, or sporozoites are administered *in ovo,* differences in the timing of the oocyst output period are observed (5). The mechanism of infection of *E. acervulina* oocysts, sporocysts, and sporozoites administered *in ovo* to embryos at different stages of late development has been investigated. Possible factors influencing the relative infectivity of different life stages administered *in ovo* include: 1) the extent of embryonic intake of life stages delivered to the amniotic fluid; 2) the ability of the embryo to process oocysts or sporocysts to the invasive stage; and 3) the ability of the sporozoite to invade and develop in the embryonic gut. Demonstration of infectivity achieved using oocysts delivered at E18 into the amnion led to experiments determining the efficacy of an *in ovo*-delivered, oocyst-based coccidiosis vaccine in broilers.

MATERIALS & METHODS

Eimeria acervulina oocysts were prepared by conventional methods and sanitized with hypochlorite. Sporocysts were released by shaking oocysts with glass beads. Intact oocysts were removed from the sporocyst preparation by Percoll™ density centrifugation (2) or filtration. Sporocysts were excysted using trypsin-taurodeoxycholate (4). Oocysts, sporocysts, and sporozoites were diluted in buffer and maintained at 4°C until use, then further diluted in buffer to the desired concentration for administration. All preparations were tested for sterility. Parasite stages of interest were administered to White Leghorn embryos or broiler embryos at different stages of development. Birds were housed in battery cages and oocyst output monitored over time, typically days two to seven post hatch. Lesion scores were determined using the methods of Johnson and Reid (3).

RESULTS AND DISCUSSION

Leghorns that received either sporozoites or sporocysts had higher oocyst output than those that received oocysts when parasite stages were administered by oral gavage to external pips at E20 (chicks had broken through the outer shell of the egg). Sporozoites were more infective than sporocysts based on oocyst output, indicating that the intestinal tissues of the E20 embryo are susceptible to infection by *E. acervulina* sporozoites. The results further indicate that the gut is capable of processing sporocysts to the

infective sporozoite stage. At E20, the limiting step appears to be rupture of the oocyst wall to release the sporocysts. It is widely believed that this occurs through muscular action of the gizzard (1).

When administered by oral gavage to leghorn embryos that had internally pipped at E19 (beak had broken through the internal egg membrane but shell was intact), sporozoites had greater infectivity than sporocysts or oocysts. This result demonstrates that the embryonic gut is capable of supporting *E. acervulina* infection as early as E19. The higher degree of infectivity of sporozoites indicates that excystation of sporocysts is not efficient in E19 embryos.

When oocysts, sporocysts, or sporozoites were administered to the amnion of leghorn embryos at 18 days of embryonic development (E18), oocysts were observed in the feces between four and seven days of age in all treatments. Although oocyst output varied for these treatments and was generally higher for sporozoites, the magnitude of the output did not always appear to correlate to the magnitude of protection in a low dose challenge model.

No hatch depression was found in either leghorns or broilers after *in ovo* administration of any of the three *Eimeria* stages in any of the experiments performed. Results indicated that *in ovo* delivery of purified oocysts, sporocysts, or sporozoites resulted in oocyst output in the fecal material collected from days two to seven post hatch. In some experiments, selective treatments were subjected to low dose homologous challenge, and partial protection was found as measured by reduction in oocyst output post challenge. In other studies, higher dose challenge of birds vaccinated *in ovo* with oocysts showed protection from lesion development when compared to non-vaccinated controls. Overall, delivery of oocysts, sporocysts, and sporozoites to the amnion of the developing embryo on day E18 does result in infectivity as ascertained by oocyst output in the hatched chick and evidence of developing protective immunity.

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INVESTIGATIONS ON DETECTION OF *HISTOMONAS MELEAGRIDIS* DNA USING PCR

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Histomoniasis, also known as blackhead, is increasingly responsible for considerable economic problems to the poultry industry, causing disastrous economic loss in chickens, turkeys and game birds. Particularly at risk are poultry reared under free range conditions. There are no products available for treatment of diseased flocks.

Under field conditions the diagnosis is mainly based on post mortem lesions. In many cases, especially in layer flocks, the conventional methods are not sufficient, since the lesions are sometimes not clear. In the laboratory the diagnosis is based on the microscopic demonstration of histomonad movement in cecal smears from freshly killed birds. Microscopic and histopathologic examination to differentiate histomonads from other flagellates, artifacts, and other microorganisms are used with some success. However, typical histomonads are not always clearly demonstrable in scrapings of infected tissue or in cecal material, and the evaluation of cecal smears can be very difficult due to the presence of other pathogenic and non-pathogenic flagellates. The technique for isolation of histomonads *in vitro* offers many advantages, but the confirmation of histomonads growing in culture may require a time-consuming procedure of rectal inoculation of culture material into chickens or turkeys. The aim of the present investigation was to establish a PCR and to examine its specificity as well as its sensitivity in the diagnosis of histomoniasis.

Histomonas meleagridis – isolate 04.3.2, which was isolated from chickens in North Georgia, was used as the positive control. A primer pair (Hm1 and Hm2) was chosen on the basis of published sequence from the *H. meleagridis* small subunit ribosomal RNA Gene (GenBank accession number AF 293056), which amplifies a fragment of 476 base pairs (bp). In trials to enhance the sensitivity using nested PCR, a further primer pair (nHm1 and nHm2) was established to amplify a fragment of 225 bp. DNA was extracted from the standard *H. meleagridis* – isolate 04.3.2, from *Trichomonas gallinae* isolated from pigeons, and from ceca and livers from field cases. From all positive samples a 476 bp fragment could be detected. On the other hand, the negative samples and the *T. gallinae* isolate revealed negative results. The trial to enhance the sensitivity using nested PCR did not resulted in an increase in the number of positive samples.

To determine the specificity a restriction enzyme analysis was carried out. The PCR products were cleaved with *Bgl*II restriction enzyme. The restriction profiles obtained by digesting the PCR products gave the calculated and expected two fragments of 342 bp and 134 bp. In addition, sequence analysis of the PCR products showed a homology of 99% with published sequence in GenBank.

For further determination of the specificity, dot blot hybridization was conducted. Only samples positive in the PCR test reacted positive in dot blot, indicating a high degree of specificity of the PCR.

The obtained results revealed that the used primers were specific and were only able to amplify DNA from histomonads and not from trichomonas. The PCR approach seems promising as a diagnostic tool in research and disease monitoring regarding *H. meleagridis*-associated disease.

BORDETELLA AVIUM: AN OPPORTUNISTIC PATHOGEN IN CHICKENS?

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ABSTRACT

Bordetellosis is one of the important upper respiratory tract diseases in poultry, especially in turkeys. It is caused by *Bordetella avium*, which colonizes ciliated epithelium and leads to inflammation and distortion of the respiratory mucosa (1). Over the recent years, there were increasing numbers of isolations of *B. avium* from the respiratory tract of chickens, broilers as well as layers, within the California Animal Health and Food Safety Laboratory System (CAHFS).

Generally, infections with *B. avium* are considered to be primarily a disease of turkeys; however, there are reports in the literature that describe upper respiratory disease caused by *B. avium* or, as it was called at the time these papers were written, *Alcaligenes faecalis* (2). In an experimental setup, there were no clinical signs directly attributed to simple *B. avium* infection, although the researchers were able to recover the bacteria for 42 days. Co-infections with Newcastle disease/infectious bronchitis vaccine, infectious laryngotracheitis vaccine, or *Mycoplasma gallisepticum* did not significantly increase the severity of disease signs or lesions. The birds used in this trial were leghorns, and the authors state that they are less susceptible to infection than broiler chickens used in a different trial (3). These findings were somewhat confirmed by another study done in North Carolina broilers where 62 % of the flocks were found to be infected during the winter months. Another finding was that *A. faecalis* was isolated more often from flocks with a history of respiratory disease compared to flocks without respiratory disease (75 vs. 29 %) (4).

In selected cases, *B. avium* was isolated from birds with a history of respiratory disease in pure culture with no other pathogens present; in other cases, isolations of *B. avium* went along with isolations of other bacteria like *B. hinzii*, *Escherichia coli*, or *Ornithobacterium rhinotracheale*. In many of the cases, the clinical signs described were those of a general upper respiratory tract infection, and typical lesions of bordetellosis were found by histopathologic examination of respiratory tract tissues, mainly the tracheas. Similar to the findings described in the literature, the severity of the disease as well as the corresponding lesions was dependant on co-infections with other pathogens like *E. coli* or *O. rhinotracheale*. Over the last 10 years, the percentage of *B. avium* isolated was between 2.1 and 8 percent, with peaks in 1997 (8.0 %) and 2003 (6.3 %).

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TURKEY RESPIRATORY DISEASE: ETIOLOGIC OCCURRENCE IN DIAGNOSTIC SUBMISSIONS

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Diseases of the respiratory system are a major cause of submissions to the California Animal Health and Food Safety (CAHFS) Laboratory System. Although diagnostic case analysis is not a true indicator of disease prevalence in the commercial turkey industry, it does illustrate relative importance of certain infectious agents. Analysis of diagnostic cases also is dependent on operations of a particular diagnostic laboratory system. Submissions to the CAHFS Laboratory System can include from one to eight birds, and test assignment and sample selection is the responsibility of the diagnostician assigned to the case. Within CAHFS, submissions from commercial poultry operations are treated on a flock basis and not as individual birds.

A ten-year retrospective analysis of turkey submissions was performed to determine what etiologic agents have been involved in respiratory diseases of turkeys. Selection criteria of submissions included necropsy performed, commercial turkeys, viral isolation or bacteriology tests were performed and respiratory sites (sinuses, trachea, lung or air sacs) were sampled. During 1993 to 2002, over 5,000 turkey necropsies consisting of about 35,000 birds were performed at the CAHFS laboratory system.

Viral isolation involving respiratory tissues and/or tissue pools resulted in 192 isolations from 660 attempts. Avian Paramyxovirus type 1 (PMV 1) and Adenovirus were most frequently isolated at 64 isolations each from tracheal samples. Twenty-three of the PMV 1 isolates had ages recorded with 15 isolates occurring between 8 to 12 weeks of age.

Escherichia coli was the most frequent bacterium isolated from respiratory tissues throughout the ten-year period and accounted for about 54% of all bacteria isolated. *Bordetella avium* was the second most frequent bacterium from 1993 to 1997, but fell to third place from 1998 through 2002. Frequency of isolation peaked in 1997 at 14% and was at a low of 6% in 2001. Fastidious gram-negative rods accounted for about 2% of the bacteria in 1993 and 1994. In 1995, Ornithobacterium rhinotrachealae (OR) was named in the CAHFS system for what had previously been called FGNR, accounted for 6% of the bacteria isolated and ranked as the fourth most frequent respiratory bacterial agent. OR ranked third during 1996 and 1997, second during 1998 and 1999, fourth in 2000, and second in 2001 and 2002. *Pasteurella multocida* was isolated with a frequency between 2 to 9% and ranked from fourth place to second place for any given year. Table 1 shows the 10 year cumulative frequency of isolation of various bacteria.

Respiratory disease in turkeys involves only a few select infectious agents. Although a number of bacteria may occasionally be encountered, most are environmental opportunists.

Table 1. Number of isolations of various bacteria from turkey respiratory samples for 10 Years.

Organism	#	%	Organism	#	%	Organism	#	%
E. coli	8105	**54%**	Klebsiella sp.	148	**1%**	Streptococcus sp.	37	**0%**
Bordetella avium	1451	**10%**	Fastidious GNR	134	**1%**	Actinobacillus sp.	35	**0%**
OR	986	**7%**	Miscellaneous	130	**1%**	Virbrio sp.	28	**0%**
P. multocida	712	**5%**	Bordetlla hinzii	94	**1%**	Neisseria sp.	27	**0%**
Pseudomonas sp.	475	**3%**	Enterococcus sp.	91	**1%**	Flavobacterium sp.	25	**0%**
Lactobacillus sp.	316	**2%**	Aspergillus sp.	83	**1%**	Alcalegenes sp.	23	**0%**
Staphylococcus sp.	294	**2%**	Pasteurella sp.	67	**0%**	Providencia sp.	10	**0%**
MM	253	**2%**	Corynebacterium sp.	66	**0%**	P. gallinarum	9	**0%**
Coliforms	235	**2%**	NFGNR	64	**0%**	Citrobacter sp.	6	**0%**
Salmonella sp.	206	**1%**	Enterobacter sp.	62	**0%**	MI	6	**0%**
P. haemolytica	205	**1%**	Erysipelothrix sp.	60	**0%**	Aeromonas sp.	4	**0%**
MG	194	**1%**	Acinetobacter sp.	45	**0%**	Candida sp.	3	**0%**
Staphylococcus aureus	163	**1%**	MS	38	**0%**	Chrysebacterium sp.	3	**0%**
Bordetella bronciseptica	154	**1%**	Salmonella arizona	37	**0%**	Serratia sp.	2	**0%**

COMPARISON OF FOUR REGIONS IN THE REPLICASE GENE OF HETEROLOGOUS INFECTIOUS BRONCHITIS VIRUS STRAINS

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ABSTRACT

Avian infectious bronchitis virus (IBV), the type member of the family *Coronaviridae*, causes an acute and highly contagious disease of chickens. Infectious bronchitis is controlled by widespread use of live vaccines containing strains of IBV from multiple serotypes. Disease outbreaks in commercial flocks occur when new viruses emerge from existing viruses through genetic changes. Analysis of the replicase gene, encoded by the 5' two-thirds of genome, is essential to fully understand the viral evolutionary process. The two polyproteins (ORF1a and 1b) encoded by the replicase gene are proteolytically processed into the smaller products required for RNA synthesis and other aspects of viral replication. Several putative functional domains, such as, a papain-like proteinase (PLP), main protease (M^{pro}), RNA-dependent RNA polymerase (RdRp), and RNA helicase encoded by the polymerase gene are important for virus replication. We have sequenced four regions of the replicase genes corresponding to the 5'-terminal sequence, PLP, M^{pro} and RdRp domains from ten heterologous IBV strains, and compared them with previously published coronavirus sequences. We found that the sequences of the M^{pro} and RdRp domains are highly conserved among coronaviruses of all groups but not the 5'-terminal sequences or PLP domains. We also found that the clustering of heterologous IBV strains based on the replicase gene do not correlate with the antigen-based S1 phylogeny. The common clustering of replicase genes from vaccine and virulent strains suggests that there has been an exchange of genetic material between these strains.

POULTRY DISEASE CONTROL: WE CAN DO BETTER

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Attendees at the XIIIth Congress of the World Veterinary Poultry Association (WVPA) were privileged to hear Dr. Erhard Kaleta, President of the WVPA, present the welcome address. Dr. Kaleta reminded the audience that *"…clubbing of farmed animals originated during severe outbreaks of a disease which is now termed rinderpest in the year 1711 in Italy. It was the pope Clement 11th who – after deliberations with some of his cardinals and medical advisors – decided to eradicate rinderpest in all cattle by clubbing and deep burying. Very soon responsible authorities in other European countries and later also in the United States of America followed this divine advice"* (2). In the first three decades of the 20th century, using this familiar approach foot and mouth disease and fowl plague were eradicated in the U.S.A. six times and twice respectively (3).

Stamping out (designation of infected zones, imposition of quarantines, destruction and disposal of infected and susceptible animals and intensive monitoring) is a well-recognized but unproven strategy for emergency livestock diseases. Certainly most veterinarians are in agreement that eliminating susceptible animals will contribute to eradication of a pathogen, but elimination of susceptible animals is not equivalent to destruction. Times have changed since the pope embarked on a rinderpest eradication campaign:

- Bacteria and viruses were discovered,
- We understand each pathogen has its own ecology,
- Our arsenal of disease control tools (antibiotics, vaccines, etc.) has expanded,
- Agriculture has evolved from a scattered poultry population disseminated throughout rural areas to dense populations on industrial farms in various rural areas,
- The consuming public has become increasingly suspicious of dramatic televised scenes of destruction and disposal of animals and
- Questions are being raised about the ethics of killing and disposing of healthy animals.

The question of whether an alternative strategy would be more effective has not been asked. In the absence of research trials to document the advantage of this archaic approach, regulatory officials should examine and document instances where emergency diseases were satisfactorily brought under control with a different approach. Low pathogenic avian influenza outbreaks have been effectively controlled by vaccination and controlled marketing as well as by stamping out, but for substantially less money. It was recently pointed out that stamping out programs for low path AI may cost 10 to 100 times more than controlled marketing (1).

Because industry-driven controlled marketing programs as well as government-driven stamping out programs have been successful, a thoughtful examination of stamping out programs leads to the idea that their success is related, not to the destruction of infected, susceptible and convalescent poultry, but to the enforced downtime, designation of infected zones, imposition of quarantines, and intensive monitoring. There is nothing special about killing and burying or burning poultry because disease outbreaks have been stopped by alternative means. Thus we can infer that it is the government's authority to quarantine, order cleaning and disinfecting, monitor and permit repopulation that accounts for its success in controlling disease.

These strengths in government programs match up well with the major weakness of industry programs. The modern poultry industry is driven by the poultry companies' needs for meat and eggs. The weakness of industry-driven disease control is that this need for a continuous supply of meat and eggs may cause companies to act in ways that do not contribute to disease control and may actually contribute to disease spread.

A new hybrid disease control program is proposed that encompasses the best that industry and government programs have to offer. Industry and government veterinarians, in a cooperative arrangement, could initiate well-thought out measures when a disease outbreak occurs.

It is no longer necessary to consider diseased or convalescent poultry as "evil." In the scientific age, we now recognize that disease control programs with totally different approaches can have the same outcome. Combining the best features of existing programs has the potential to improve the existing disease control strategies, to reduce the objections that have been raised about them and to reduce disease control costs.

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EVALUATING INVESTMENT IN ENHANCED BIOSECURITY FOR EGG PRODUCTION OPERATION

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SUMMARY

Enhancing structural and operational biosecurity for US egg-production units will require investment in capital improvements and expenditure for additional labor and consumables. A quantitative approach to justifying investment involves assessing the risk and consequences of infection. In the given case, appropriate capital improvements costing $133,000 were required with a projection of annual operating costs, including both fixed and variable components of $77,310. This value represents an incremental cost of 0.52¢ per dozen over a projected annual output of 14.7 million dozen eggs from a complement of 650,000 hens. The net present value of savings associated with averting a catastrophic infection such as END range from $2.3 million, assuming one infection in 10 years to a loss of $265,000 with a 10% probability of an infection occurring over a 10-year period (p=0.1). At the highest probability of infection (p=1.0), the ratio of the present value of savings to initial capital investment would be 17.2, using an 8% discount factor. In contrast, an erosive disease such as LPAI would incur a cost of $204,000 per outbreak and would not justify the cost of a comprehensive program of structural and operational biosecurity.

Decisions relating to the erection and installation of capital improvements and implementation of operational procedures to restrict introduction and dissemination of disease should be based on a projection of the cost of protective measures, their efficiency, the probability and consequences of infections and the microeconomic factors pertaining to the subject enterprise.

INTRODUCTION

Recent outbreaks of exotic Newcastle disease (END) and avian influenza of low-pathogenicity (LPAI) have stimulated interest in enhancing biosecurity in the egg-production industry. A number of factors inherent to the structure of the industry increase vulnerability to introduction of disease and the dissemination of pathogens within complexes. (1) In-line operations house up to 1.5 million hens at a single location with an age-spread ranging from 16 through 120 weeks. Many in-line processing plants receive nest-run eggs from other farms and extensive intra- and inter-company trading of eggs represent a potential for infection. A significant risk is represented by purchase of feed from plants which deliver to multi-species and diverse poultry operations in vehicles which are inadequately decontaminated. Justifying expenditure on structural and operational biosecurity for a complex should be based on an analysis of the epidemiology of specific diseases, probability of their occurrence in the area of operation, financial consequences of an outbreak, and a projection of the discounted benefits attributable to a program of prevention (2).

A study was undertaken to evaluate the feasibility of enhancing biosecurity for an in-line complex comprising 650,000 hens located in a mid-Atlantic state.

METHODOLOGY

The following sequence of analysis was followed:

- Define diseases of financial significance which can be partly or completely prevented by intensified structural and operational biosecurity or a combination of biosecurity measures and immunization.
- Assess the risk of introducing specified diseases based on their epidemiology in relation to the subject enterprise.
- Quantify the impact of catastrophic diseases and infections of intermediate and mild severity.

- Project the financial loss associated with individual diseases or their combination
- Identify and prioritize potential sources of infection for specified diseases including purchase of nest-run eggs, feed delivery vehicles, work crews and equipment to move flocks, authorized visitors and trespassers, contact with free-living birds and introduction by the air-borne route.
- Design improvements to enhance structural biosecurity to protect against specified disease risks.
- Develop a program of operational biosecurity appropriate to risk of infection which is consistent with available facilities reflecting the best practices of successful industries confronted with endemic diseases.
- Determine the return derived from capital and operating expenditure required to improve biosecurity. Discounted cash-flow analysis is required to quantify benefits derived from savings accruing through averting disease over a defined time period following investment in improvements.

RESULTS AND DISCUSSION

Installations required. A practical level of biosecurity for the subject farm would require erection of a change-room and shower module, a decontamination facility for vehicles, a gatehouse at the entrance to the farm, perimeter fencing for the live-bird production area and upgrading of security to prevent unauthorized entry to houses. The proposed improvements in structural biosecurity would provide a high level of protection against catastrophic diseases, providing that commensurate operational biosecurity procedures including immunization, where appropriate, are followed (3). The fixed capital cost of designated improvements was estimated to be $133,000.

Operating expenses included annual fixed costs comprising depreciation, interest and an overhead provision, were calculated to be $25,610. Annual operating costs including incremental labor, maintenance, microbiological and serologic monitoring and disease surveillance amounted to $51,700. The comprehensive program would generate a total annual cost of $77,310 for enhanced biosecurity equivalent to 0.52¢/dozen over an annual production of 14.7 million dozen eggs.

Evaluation of the benefits of protection against a catastrophic disease. A major component of the losses following introduction of END is derived from the difference between Federal or State compensation for hens depleted and their actual commercial value. This was estimated to be $1.3 million. A minimum 30-day period of depletion would be followed by phased replacement of flocks at 8-week intervals, impacting production over 52 weeks. The loss in saleable product would range from zero at the time of depletion to 40,000 dozen per day 52 weeks after infection. These values are based on a complement of 650,000 hens with an average production of 78% and 95% saleable production. The average daily loss of 20,000 dozen over a 52-week period would total $1.1 million until full production was attained.

Additional costs associated with decontamination and disruption of operations would amount to $250,000. Losses following an outbreak of END or HPAI, necessitating depletion, would therefore amount to $2,648,500. In the case of the subject enterprise, the affiliated feed mill would experience an increase in unit fixed production cost of $3/ton as a result of decreased output. This would add an additional $450,000 loss to the enterprise during the year following infection. Intangible losses including degradation of brand value from sale of premium eggs and erosion of goodwill could raise the cost of a single catastrophic infection to $4 million.

Evaluation of benefits of enhanced biosecurity. An annual outlay of $77,310 would be incurred if a comprehensive level of biosecurity were to be implemented over a 10-year period. The benefits from the program were considered in relation to the probability of infection expressed as a continuum extending from zero, indicating no exposure, to 1.0 or absolute exposure over a decade.

If it is accepted that one infection with END will occur during a 10-year period (p=1), the loss experienced as a result of an outbreak would be $4 million. This could be prevented by an outlay of $773,100, the sum total of 10 consecutive years of the program. The net savings represented by the difference between projected loss and the outlay on biosecurity is $3,226,900. Since the evaluation considers a 10-year period, calculation of the time-related benefit is simplified by assuming that the disease occurs during the fifth year of the period. Accordingly, the difference between savings and expenditure is discounted by a factor of 0.71, reflecting the 8% cost of capital. The present value of the savings accruing from a single outbreak in 10 years is therefore $2,291,099. The discounted benefit-to-cost ratio of 17 justifies the initial capital investment of $133,000 in enhanced structural biosecurity.

The evaluation was repeated with probability values of p = 0.75, 0.5, 0.25 and 0.1for a disease occurring in 10 years as shown in Table 1. It can be seen that the discounted savings as a result of preventing END decline from $2,291,099 with absolute certainty of one outbreak in 10 years (p = 1) to a break-even probability of approximately 0.2. If there is only a

1 in 10 chance of an outbreak in 10 years (p = 0.1), the investment in enhanced structural and operational biosecurity will result in a loss of $264,901.

Evaluation of an erosive disease. In order to evaluate the benefits of enhanced biosecurity against an erosive disease, calculations were performed to determine the savings associated with averting an outbreak of LPAI. The costs associated with introduction of this disease are based on industry experience in Connecticut following introduction of the H7N2 strain.

The projected loss in egg production would amount to an average of 15% over 28 days. In a flock of 650,000 hens this would amount to 168,560 dozen over the period. Purchasing nest-run eggs to compensate for the projected decline in production would involve expenditure of $101,136 at a replacement cost of 60¢/dozen. The impact of LPAI would be proportional to the prevailing cost of eggs. It is noted that in the event of any regional or national outbreak of disease, egg prices rise both in response to a disturbance in the supply to demand equilibrium but also due to the perception of shortages among buyers for retail chains and institutions.

Assuming that prevailing State regulations mandate administration of an inactivated homologous AI emulsion, $53,125 would be required to cover the cost of purchase and administration of vaccine.

Immunization of replacement pullets transferred to the subject farm would continue with varying intensity for up to 5 years at a cost of $50,000. The direct and indirect costs of a LPAI outbreak were calculated to be $204,261 for the subject enterprise.

It is noted that the saving accruing from preventing a single outbreak of a disease of low pathogenicity over a 10-year period is less than the expenditure on enhanced biosecurity. Theoretically the comprehensive program would not be justified to prevent an erosive disease such as LPAI. This calculation does not take into account intangible losses including disruption of operations, quarantine, reduced feed tonnage and degradation of goodwill. Intensive biosecurity procedures are not financially feasible to prevent losses associated with LPAI, assuming that State control measures will allow vaccination with minimal quarantine restrictions.

Applying a feedback approach to evaluating the cost of protection and benefits facilitates reduction in expenditure on structural biosecurity, commensurate with the consequences of infection. Elimination of a decontamination module for vehicles, together with corresponding economies in operational biosecurity, could reduce the cost of a program to establish a breakeven point corresponding to a probability of infection of approximately 0.5, representing a 50% chance of an LPAI outbreak during a 10-year period. If the decision is made to upgrade biosecurity for strategic reasons to protect against a catastrophic disease, or is required by a lending institution, the need to justify protection against an erosive infection is moot.

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Table 1. Present value of savings associated with enhanced biosecurity against exposure to END.

Probability of a catastrophic disease outbreak over 10 years	Discounted Value of Savings/ (Loss)	Ratio of Present Value of Savings to Investment
1.00	$2,291,099	17.2
.75	$1,581,099	11.9
.50	$ 871,099	6.5
.25	$ 161,099	1.2
.10	$ (264,901)	(2.0)

CONTEMPORARY POULTRY HOUSING IN ENGLAND AND GERMANY

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ABSTRACT

In July of 2002, UEP (United Egg Producers) organized a trip to the countries of England and Germany for the purpose of allowing US industry persons to observe the current housing types and philosophies of our counterparts in Europe. The direction of the European industry is for the "humane care" and "enrichment" for the birds they care for. This philosophy is being promoted and organized by UEP currently in the United States. Types of houses will be shown and I will demonstrate a few of the methods by which they attempt to achieve that goal.

EFFICACY OF A *HAEMOPHILUS PARAGALLINARUM* BACTERIN IN A NOVEL OIL EMULSION

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ABSTRACT

Haemophilus paragallinarum is the etiology of infectious coryza, a highly contagious, acute respiratory disease of poultry. This disease can be economically devastating in tropical and subtropical parts of the world. Infected laying flocks can have a reduction of egg production by 10-40%. *H. paragallinarum* isolates were originally grouped into serovars by Page, designated A, B, or C. Serovar distinction remains critical to discussions of bacterins since only homologous protection is known to exist.

The bacterins produced against *H. paragallinarum* have the ability to exhibit adverse post vaccination reactions, especially those in water-in-oil emulsions. These reactions can result in decreased feed consumption, a delay in sexual maturity, and a reduction total number of eggs produced per hen. In general the bacterins that are water-based in aluminum hydroxide offer less post-vaccination reaction, but protection can be reduced. The ideal vaccine would include all three serovars incorporated into an adjuvant that delivered the antigens to the immune system stimulating a long-lived, high level of immunity without adverse reactions.

An experimental vaccine using a novel adjuvant containing the three serovars of *H. paragallinarum* has been tested against a commonly used water based commercial vaccine also containing all three serovars. Previous field experience with this commercial water based vaccine has demonstrated the desirable minimum post vaccination reaction. Groups of three-week old specific pathogen free (SPF) birds were vaccinated subcutaneously with either the experimental vaccine or the commercial water based vaccine. Three weeks post vaccination, the vaccination sites of the birds were evaluated for adverse reaction and scored numerically with a standardized lesion scoring system. The experimental vaccine produced an average injection site reaction score of 0.63 compared to the commercial water based vaccine's higher score of 1.9.

A challenge trial was completed to prove efficacy of the product in the novel emulsion. One hundred and fifty SPF chickens, five weeks of age, were divided into six groups. Three groups were vaccinated subcutaneously in the neck with one dose (0.5 ml) of the experimental bacterin and revaccinated four weeks after the initial vaccination. The remaining three groups served as non-vaccinated, challenged controls. At two weeks post revaccination all birds were challenged intranasally with one of the three challenge strains of *H. paragallinarum.* Challenge dose varied by serovar, see table 1. The chickens were observed daily for signs of facial swelling or nasal exudates for a period of 10 days post-challenge. All birds were housed under conditions of high temperature and high humidity throughout the observation period. Chickens exhibiting two consecutive days of facial swelling and/or two consecutive days of nasal exudates during the observation period were considered positive for *H. paragallinarum* infection. Results are found in Table 1.

Duration of immunity challenge studies are currently underway and results should be available by the time of the conference but were not available at the printing of the proceedings.

Table 1. Percent Protection following intranasal challenge.

	Serovar Challenge	% Protection
Vaccinated	A (8.46 x 10^7 CFU)	100%
Non-Vaccinated	A	28.6%
Vaccinated	B (5.64 x 10^5 CFU)	91.7%
Non-Vaccinated	B	20.0%
Vaccinated	C (8.64 x 10^7 CFU)	93.8%
Non-Vaccinated	C	5.6%

IMMUNOGENIC AND CROSS-PROTECTION STUDIES OF THE NINE SEROVARS OF *HAEMOPHILUS PARAGALLINARUM*

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ABSTRACT

Infectious coryza is an upper respiratory disease of chickens caused by *Haemophilus paragallinarum*. Two related schemes have been used to serotype this bacterium. The Page scheme recognizes the three serovars, A, B, and C. The Kume serotyping scheme recognizes nine serovars distributed into three serogroups. Thus, the nine currently recognized serovars are termed A-1, A-2, A-3, A-4 (all of which correspond to Page A), B-1 (which correspond to Page serovar B), C-1, C-2, C-3, and C-4 (all of which correspond to Page serovar C) (2). It is generally accepted that the Page serovars, or Kume serogroups, represent three distinct immunovars. Thus, the aim of the present study was to investigate the cross-protection and hemagglutination-inhibition antibodies afforded in vaccinated and challenged chickens with the nine serovars of *H. paragallinarum*.

The nine reference strains of the Kume scheme were used in the present study. These strains are sourced from several countries and some of them are geographically restricted: A-3 in Brazil, C-1 in Japan, C-3 in South Africa and Zimbabwe, and A-4 and C-4 in Australia. The remaining strains are widely distributed (3).

Obtained results confirmed the widely accepted dogma that serogroups A, B, and C represent three immunovars. Particularly, our results confirmed previous findings that a C-2 vaccine protects against C-1 and C-4 (1). Within a serogroup it appears that there is significant cross-protection between serovars. However, partial protection was recorded between some serovars. Similarly, interesting findings in serology were observed. This is evidence that antigenic differences are highly significant in terms of immunity.

In conclusion, this study could explain outbreaks of infectious coryza observed in vaccinated flocks. Furthermore, our results and distribution of *H. paragallinarum* serovars indicate that most areas would be best protected by a vaccine containing strains A-1, B-1, and C-2.

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ANTIBODY PRODUCTION AND PERSISTENCE OF MAREK'S DISEASE VIRUS IN VACCINATED CHICKENS

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ABSTRACT

Antigen extracted from cell cultures infected with serotype 2 Marek's disease (MD) vaccine virus was obtained from a commercial source for the agar gel immunodiffusion (AGID) test for MD. This same antigen was also used for an enzyme-linked immunosorbent assay (ELISA-a). For comparative purposes, ELISA-b was conducted with whole cells infected with serotype 1, 2 or 3 vaccine virus as antigen. Commercial White Leghorn chicks were vaccinated at hatch with serotype 1, 2 or 3 virus and were challenged with the highly virulent RB1B isolate of MD virus (serotype 1) at 21 days of age. Chickens were bled and killed at intervals post challenge and tissues were collected. Serum was tested for antibody and the tissues were tested for virus by polymerase chain reaction (PCR) techniques. The AGID test detected antibody to RB1B isolate of MD virus and to all vaccine viruses, but reactions were strongest with sera from chickens infected with MD and serotype 2 virus. ELISA-a and AGID did not always detect antibody in the same chicken. In one experiment, ELISA-a detected maternal antibody in 12 of 16 chicks on day 21 whereas none were positive by AGID. On the other hand, actively acquired antibody to vaccines was detected earlier by AGID than by ELISA-a. ELISA-b detected antibody against all 3 serotypes of virus and showed that there was considerable cross reactivity. The combined results from these serological tests showed that all three serotypes of vaccine elicited a strong humoral immune response but based on PCR tests for virus in the spleen, serotype 2 was less effective than serotypes 1 and 3 in reducing infection with virulent MD virus.

CALIFORNIA EXPERIENCE WITH EXOTIC NEWCASTLE DISEASE: A STATE AND FEDERAL REGULATORY PERSPECTIVE

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Discovery

- Exotic Newcastle Disease (END) was first diagnosed in California when dead birds from backyard flocks in Compton and Montebello in Los Angeles County and Norco in Riverside County were submitted for examination to the California Animal Health and Food Safety Laboratory.
- The United States Department of Agriculture (USDA) confirmed the disease at the Compton site on October 1, 2003. The other sites were also later confirmed.
- A task force was immediately formed with the California Department of Food and Agriculture (CDFA) and USDA staff.

Timeline

- Diagnosis......................................October 2002
- State of emergency declared .... January 2002
- Eighty-four percent release of quarantine .... August 2003
- Complete release of quarantine .September 2003
- From discovery to eradication............ 11 months

Estimated PROGRAM Costs

- California..$161 million
- Nevada..$ 6 million
- Texas ....$ 4 million
- Arizona...$ 4 million

Estimated INDEMNITY Costs

- U.S...$ 23 million
- California......................................$ 22.6 million
- Nevada...$274,000
- Texas $164,000
- Arizona...$ 40,000

Operations Report (Totals)

- Birds depopulated............................ 3.16 million
- Backyard birds depopulated (of the total above)...145,000
- Premises quarantined................................18,435
- Infected or exposed premises 920
- Backyard flocks destroyed 899
- Commercial flocks destroyed..........................22

Value of Commercial Poultry in California

- Annual value .... $1.3 billion
- Number of layer birds......................... 24 million
- Egg production .... 6 billion
- Number of broilers .... 235 million
- Number of turkeys........................... 17.7 million

Trade Impacts

- Trade restrictions resulting from the disease had negative impacts on both California and U.S. poultry and egg producers.
- The following countries had imposed various trade restrictions on poultry and products from California: Japan, Taiwan, Canada, Tahiti, Poland, Korea, Bulgaria, Hungary, Iran, Philippines, Morocco, Romania, Russia, Saudi Arabia, China, Mexico, Azerbaijan, Guatemala, Latvia, Lithuania, Mauritius, New Caledonia, Switzerland, Indonesia, Thailand, and Western Samoa.
- The following countries had imposed various trade restrictions on the U.S. poultry industry: European Union, Argentina, Guam, Colombia, Grenada, Jordan and Uruguay.

Social Impacts

- Commercial operators, processors, feed mills and other allied industries were predictably interested in cooperation because they had a good understanding of the disease and a desire to resume business.
- Pet bird owners, commercial pet bird breeders, pet stores, swap meets, auctions, small animal veterinarians, and animal shelters presented special challenges and varying degrees of cooperation. Most people cooperated in order to minimize the overall impact of the disease. In fact, individual efforts to improve biosecurity and control movement of birds, especially free-roaming neighborhood poultry, were imperative to the ultimate effectiveness of the program. Even with this general level of cooperation, social impacts were significant, because many of the birds destroyed had value to the owner well beyond "fair market value," and many of the businesses effected had no way to recover losses. Further, many individuals were frustrated by the communication challenges inherent in a large, dynamic task force, and a few people objected to the government's authority to take firm action to eradicate diseases like END.
- Another significant social aspect of the outbreak involved game fowl owners. While owning, breeding, showing and selling these birds is not illegal in California, fighting them, or owning them with the intent to fight, is illegal, making parts of this population difficult to identify. Game fowl enthusiasts are a well-established part of the "rural-urban" interface. Backyards, horse stables, vacant lots, and small warehouses can be found containing poultry in many parts of what would appear to be an otherwise urban or suburban area. These small facilities are greatly dispersed throughout the Los Angeles basin. The cooperative care, sale and movement of these birds contributed to the extremely rapid spread of the disease.

Response: Quarantine Zone

- At the peak of the outbreak, counties under quarantine were Imperial, Kern, Los Angeles, Orange, Riverside, Santa Barbara, San Bernardino, San Diego and Ventura.
- California's quarantine zone covered 46,000 square miles.
- END was also found in Clark County, Nevada; La Paz County, Arizona, and El Paso County, Texas.

Response: Task Force Personnel

- The Incident Command System provided organizational structure for task force personnel. This is a flexible system designed to help managers respond to emergencies of essentially any type, size or complexity.
- In total, the task force had over 7,000 individuals rotating in and out over the course of the 11-month quarantine.
- These employees worked more than 256,000 days. This represents nearly 1,000 person

years of labor, mostly at 12 hours per day, 7 days per week.
- The END task force included the expertise from 10 major state and federal agencies as well as seven units within USDA's Animal and Plant Health Inspection Services. It also included many city and county agencies.
- No single agency could have dealt successfully with END on its own.

Response: California Task Force Partner Organizations

- U.S. Department of Agriculture
- California Department of Food and Agriculture
- Governor's Office of Emergency Services
- California Animal Health and Food Safety Laboratory
- U.C. Cooperative Extension
- California Conservation Corps
- California Department of Fish and Game
- California Department of Forest and Fire Prevention
- U.S. Forest Service
- California Department of Health Services
- California Department of Mental Health
- California Environmental Protection Agency (DPR, IWMB, WQCB)
- California Department of Transportation
- California Highway Patrol
- Commercial poultry companies and trade associations, allied industry
- Local: agricultural commissioners, law enforcement, animal control, health departments, government officials
- Private: veterinarians, bird clubs and organizations, humane societies, community activists

Response: Outreach

- Task force public information staff provided weekly updates to approximately 65 media outlets in California. Information officers handled an average of 10 media calls per day, but a busy day could bring in as many as 30 media calls.
- The task force produced and distributed a PSA that ran on TV stations throughout Southern California.
- Other information dissemination: through the Office of Emergency Services network, commercial poultry liaison, feed stores, town hall meetings, community groups, mass mailing, door to door personal visits, flyers, industry groups and organizations, websites, and racing pigeon, commercial poultry, and pet bird END task force advisory groups.

Response: Hotline

- A telephone hotline for questions and information was established during the first week of October. The hotline continues in operation to this day.
- The number is 1-800-491-1899.
- Attendants are bilingual in English and Spanish.
- Hours of operation are from 7:00am to 8:00pm, Monday through Friday, and from 8:00am to 4:30pm on weekends.
- Recorded information is available 24/7 in English, Spanish and Vietnamese.

Response: "In the Field"

- Veterinarians examined birds for clinical signs of END; if present, the property was quarantined and birds were tested. If positive, the birds were euthanized using CO2 gas.
- Task force members then conducted door-to-door surveys to identify birds in a radius of one kilometer to determine if there were additional infections or exposure and to quarantine properties in order to stop bird and related equipment movement.
- If epidemiologists decided that there was exposure to the disease on other properties, then the exposed birds were euthanized or placed under premises quarantine. This depended on the circumstances and a risk assessment.
- All depopulated birds (infected and exposed) were appraised and owners were compensated with an indemnity payment.
- All affected areas were thoroughly cleaned and disinfected. Landfills, composting and rendering were used for safe disposal of affected birds and materials.
- Following disinfection, sentinel birds may be introduced to test for the virus.
- The quarantine was lifted after assurance that each property was clean and the surrounding area was free of disease.

Source of Outbreak?

- The source remains under investigation. It could have been carried into California on contaminated equipment, people or products. An infected bird smuggled into the state also could have introduced it.
- The END virus discovered in California in 2002 is genetically similar to the END virus found in Mexico in 2000.

Preventative Measures: Avian Health Program

- The Avian Health Program is currently being developed to prevent or minimize another avian disease outbreak in California. Similar, complimentary efforts are taking place at a national level.
- The programs involve active and passive surveillance and public education.
- A team of State and Federal personnel remains in Southern California to develop and implement mitigation measures. Projects include outreach on feed bags, calendars with biosecurity messages (three types - game fowl, fancy poultry, pet birds), biosecurity videos for backyard poultry, feed store owner biosecurity training and certification, game fowl breeder health assurance program and certification, swap meet vendor biosecurity training, active surveillance in swap meets and auctions, commercial poultry operations, custom slaughter plants, game fowl breeding flocks, and animal shelters, on-going bilingual biosecurity training for commercial poultry workers, biosecurity training for law enforcement and animal control agencies, and community avian health training programs (disease, vaccination, laboratory services, biosecurity).
- Information is available on the Internet about the Avian Health Program at www.cdfa.ca.gov.
- Besides Avian Health Group efforts in Southern California, ten counties in Northern and Central California have one-year cooperative agreements with the task force to monitor for the disease and conduct public outreach (Fresno, Kings, Merced, Placer, Sacramento, San Joaquin, Sonoma, Stanislaus, Tulare and Tuolumne).

Historical Note: Last Outbreak of END

- The last major outbreak of END in poultry in the United States occurred in California in the 1970s (1971-1974). It was eradicated at a cost of $56 million (1973 dollars).
- That outbreak affected 1,341 flocks in eight Southern California counties.
- A total of 11.9 million birds were euthanized.
- Vaccination was used and eventually abandoned as it minimized the clinical effects of the disease but did not prevent infection from spreading.

EFFECT OF DIET ON ALPHA TOXIN PRODUCTION BY *CLOSTRIDIUM PERFRINGENS* TYPE A

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ABSTRACT

Wheat supernatant (W) and corn supernatant (C) were derived from *in vitro* digestion of these cereals to mimic that of an avian digestive process. An examination of alpha toxin production was carried out when, in three separate trials, *Clostridium perfringens* type A (CPA) was grown in either W or C combined with thioglycollate (TG); TG; and TG plus pepsin (60,000 units, Sigma) and pancreatin (59.1 mg, 8 x U.S.P., Sigma) (TGE). 100 ul of a pure CPA culture was placed into either 2 ml W plus 4 ml TG; 2 ml C plus 4 ml TG; 6 ml TG or 6 ml TGE, and incubated anaerobically at 40°C for 4 hr. Serial dilutions were performed, and colony forming units (CFU) per ml were counted from blood agar plates. In each of the three trials, there were significantly more CFU per ml of CPA when grown in W ($1.05x10^{9}$, $1.18x10^{9}$, $1.52x10^{9}$) compared to C ($3.6x10^{8}$, $5.85x10^{8}$, $8.83x10^{8}$), TG ($3.58x10^{8}$, $2.85x10^{8}$, $8.1x10^{8}$) and TGE ($4.5x10^{8}$, $4.15x10^{8}$, $4.7x10^{8}$) respectively. To determine alpha toxin production, the remaining culture was centrifuged to remove bacteria and 1.8 ml placed in 10K Microsep Omega centrifugation filters and centrifuged for 60 to 165 minutes. Alpha toxin was measured from the volume-equalized retentate using the Amplex Red phosphatidylcholine-specific phospholipase C assay kit (A-12218). Units (U) of alpha toxin per CFU were determined, and log values were subjected to a one way analysis of variance with differences considered significant if $p<0.05$. Log mean alpha toxin production (U/CFU) was significantly

higher in C (-6.64) compared to TGE (-6.98), TG (-7.03) and W (-7.27). TGE was significantly higher than W. There was no significant difference between TGE and TG, nor between TG and W. In conclusion, it was found that there was significantly more proliferation (CFU/ml) when CPA was grown in W compared C. However, alpha toxin production (U/CFU) was significantly higher when CPA was grown in C compared to W.

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

CHARACTERISTICS OF CAPSID SEQUENCE IN PIGEON CIRCORVIRUS

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A Columbid circovirus-like agent was reported in 1993 by Woods, *et al*. (1). A moderate amount of information is known on the virology of Columbid circovirus (CoCV), however, little information is known of the prevalence and variability of the virus. Bursal tissues were tested for CoCV by a PCR assay to determine the prevalence of the virus among numerous commercial squab producers in the Central Valley of California. The capsid gene from a small number of pigeons was amplified and sequenced. Comparisons of the nucleic acid sequences of the capsid protein from individual birds in a single submission and sequences from different submissions were performed to determine the variability of the virus among these commercial producers and non-commercial pigeons in California. Preliminary results of these comparisons show one or two related strains within a single producer and closely related strains among producers. Additional results will be presented.

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COCCIDIAL LESIONS IN COMMERCIAL FLOCKS VACCINATED WITH A COCCIDIOSIS VACCINE

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ABSTRACT

Lesions seen with the use of coccidiosis vaccines generally appear starting at about 14 days post vaccination. By 35 days post vaccination all gross lesions have resolved. Contrary to this, on most coccidiostat programs lesions generally start appearing at about 35 days. The first flock of birds vaccinated with a coccidiosis vaccine following a coccidiostat program experience a greater percentage of necropsied birds exhibiting gross lesions. The severity of the lesions, based on the Johnson and Reid method of lesion scoring are not more, only in incidence of lesions. With successive flocks on the vaccine program the "percentage incidence" of lesions decreases. It is presumed the decrease in incidence is the result of a decrease challenge. This decrease is believed to be a result of replacement of field strain oocyst with vaccine strain oocyst.

The gross lesion scores for *E. acervulina*, *E. maxima*, and *E. tenella* typically all fall with in the range of 1+ to 2+, based on the Johnson and Reid Method.

PODODERMATITIS IN MEAT TURKEYS

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Pododermatitis is defined as inflammation of the footpad, characterized by swelling of the foot and erosive lesions on the footpads and/or digits. Various causes may be responsible for the footpad lesions, including stocking density, type of litter, litter management and condition, and nutritional factors such as biotin deficiency (2).

In the winter-spring of 2001 several turkey submissions were made to the California Animal Health and Food Safety Laboratory System, Fresno Branch with a history of shaky legs, down birds, and swollen footpads. Submissions were made from two different producers. Producer 1 kept the birds indoors, while birds from the producer 2 were kept outdoors after the initial brooding period. All submitted turkeys were males, between 15 and 22 weeks of age.

At necropsy, most birds had severe swelling and hardening of the footpads. The skin of the footpad was thickened and black. On cross section of the foot, there was abundant connective tissue in the subcutaneous tissue. There was no exudate or caseous material in the footpad. Similar changes were also observed on the ventral portion of the second digit. Breast blisters were also observed in 25% of these turkeys, and swelling of the hock was seen in 5% or the birds. Histology of the footpads revealed focal areas of necrosis of the epithelium. Bacteria were only seen on the surface of the necrotic areas of the skin. At the margins of necrotic areas, the epithelium was mildly vacuolated. Abundant connective tissue with large amounts of fibrous tissue and neovascularization were observed in the subcutis. Bacteria, including *Actinomyces* sp., *E. coli*, and *Staphylococcus* sp., were isolated occasionally and in low numbers.

The skin of unaffected areas of the footpad had normal epidermal structure with a stratum basale, spinosum, transitivus, and corneum, unlike in biotin deficient birds in which these layers are poorly differentiated (1). Birds from all these flocks have been submitted previously to the laboratory and were diagnosed with enteritis. Diarrhea may have caused excess humidity in the litter that is hard to dry during the cold and humid months of the year. Furthermore due to high cost of the litter, producer 1 recycled the litter. Male turkeys are kept in houses for longer and therefore are exposed to these adverse conditions for a longer time. Ventilation system has been considered a predisposing factor for pododermatitis (3); however, producer 2 kept birds outdoors. Finally, higher weight in male turkeys causes more pressure on their footpads. Poor litter quality due to excess humidity and caked litter were the most likely etiology of these problems.

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FOOT PAD DERMATITIS AND WELFARE IN ITALIAN BROILER CHICKENS

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ABSTRACT

An attempt to classify broiler foot health status using the method developed in Sweden (2) for the control of foot pad dermatitis was carried out. Italian market demands heavy broilers (3.5 kg and more), at 56 days of age. The foot pad dermatitis is not frequent and it is correlated to feed composition, litter material, stocking density and climatic conditions. Evaluation and classification of foot pad dermatitis at slaughterhouse showed that gross and histological lesions were not always comparable.

INTRODUCTION

Foot pad dermatitis has long been a neglected problem in broiler production. Recently Boards of Agriculture in some countries of Europe proposed a program in order to classify broiler foot health status as an index of rearing conditions and chickens welfare (2,5). Foot pad dermatitis is a type of contact dermatitis affecting the plantar region of the feet. In an early stage, discoloration of the skin is seen. Hyperkeratosis and necrosis of the epidermis can be seen histologically. In severe cases the erosions are developed into ulcerations with inflammatory reactions of the subcutaneous tissue. Although not primarily caused by any particular microbial agent, the lesions often become infected by a variety of bacteria and fungi, especially *Staphylococcus* spp. (1).

MATERIALS AND METHODS

In the last spring investigations on foot pad dermatitis carried out in 100,000 broilers 50 (± 6) days old at slaughterhouse. One leg for 100 chickens/slaughtered group was controlled. Lesion score indicated by Ekstrand *et al.* (3) was used: score 0 = no visible lesions or mild hyperkeratosis, score 1 = erosion in the epidermal layer > 1 cm, score 2 = severe ulcerations. The total foot pad score per flock was calculated as follows: Σ = score 0 x 0 + score 1 x 0.5 + score 2 x 2. Lesions were histologically observed. Ekstrand *et al.* (4) arbitrarily selected the value of 40 to show the quality of chickens welfare. If final group score is over 40 the maximum stocking density must be decreased from –1 to –3 kg/m^2.

RESULTS AND DISCUSSION

The total foot pad score per flocks has been lower than 40, because the climate (spring) and litter (wood-shavings) in monitored flocks were very good. The classification of the lesions during the slaughtering is impossible. Gross and histological lesion in the same sample was not always comparable. Microscopic examination shows more severe lesion. In the future we will check foot pad dermatitis in several broiler farms characterized by different litters, age of slaughtering and climatic conditions.

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CONSIDERATIONS TO CONTROL AVIAN INFLUENZA

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Through time the avian influenza viruses have proven to be a constant threat to human beings and animals because they mutate very frequently and have constant genetic movement, such as the recent different subtypes of influenza virus: H5N1 in Hong Kong; H7N7 in Holland, Belgium, Germany; H7N1 in Italy; H7N2 in USA. The OIE consider the subtypes H5 and H7 most dangerous to chickens. In May 1994 avian influenza (AIV) was reported in Mexico. Since that time the government and poultry industry have been taking different measures to control and eradicate the disease. One approach is vaccination. The Mexican government authorized the inactivated vaccine in 1995. The Mexican government provides the master seed (A/Chicken/Mexico/232/94/) (H5N2) and manufacturing laboratories; additionally, the recombinant vaccine was authorized. Despite the fact millions of chickens have been vaccinated with both vaccines and many scheduled vaccinations, the low pathogenic AIV continued circulating within the environment. The main reasons are:

1. The time between the last flock and the new one is short. This situation induces inadequate disinfection and incites the virus to permanently remain on the farm. Cold weather (winter and the beginning of spring) contributes to this.

2. The presence of maternal antibodies in the progeny is very important. The majority of the breeders are vaccinated, and so their progeny have humoral antibodies. In the trials that we made we watched the catabolism of the antibodies finish around the fourteenth day of age. It does not matter if the progeny have high or low amounts of antibodies to AIV. The maternal antibodies will block the virus from the vaccine in part, if the vaccine is applied at an early age, the amount of antibodies in these chickens are less compared with chickens with low maternal antibodies at the time of vaccination. The immune response will be appropriate if the vaccine is applied at 10 days of age in chickens with maternal antibodies. We had proved it.

3. The chickens show variations in the immune response depending on which laboratories manufactured the vaccine, and in some cases between batches within the same laboratories.

4. The chickens exposed to the AIV in early ages (2 or 3 weeks of age).

5. The flock constantly has contact with the AIV because they exist in zones of high risk. In these areas the poultry companies have different systems at work.

Table 1. Inmunization scheme.

Maternal Antibodies	Exposition of Age (weeks)	Risk Area	Vaccination					
			First shot			Booster		
			Age (days)	Type	Dose	Age (days)	Type	Dose
No	2 to 3	Yes	1	Gel	0.3 ml	16	Oil	0.5 ml
Yes	2 to 3	Yes	6 Gel al 4%		0.5 ml Hiperc.	16	Oil	0.5ml
No	3 to 4	Yes	1	Gel	0.3ml Hiperc	18	Oil	0.5ml
Yes	3 to 4	Yes	10	Gel	1ml.	18	Oil	0.5ml
Yes	3 to 4	No	10	Gel	1ml.	No	No	No
No	4 to 5	Yes	1	Oil	0.3ml hiperc	22	Oil	0.5ml
Yes	4 to 5	Yes	10	Oil	1ml	22	Oil	0.5 ml
Yes	4 to 5	No	10	Oil	1ml	No	No	No
Yes	5 to 6	Yes	12	Oil	0.5ml	No	No	No
No	5 to 6	Yes	8	Oil	0.5ml	No	No	No
No	5 to 6	No	8	Oil	0.5 ml	No	No	No

The choice criteria in these schedules will rely on the value of the flock, the season, weather, and the risk area.

SUGGESTIONS

1. In some poultry farms, the chickens receive two doses of influenza vaccine at different ages. Depending on whether the farm is in risk area, we suggest only the first injection with influenza virus alone, and do not combine with others antigens like Newcastle.
2. The vaccination crew will receive continuous training and supervise the process and progress of vaccination. We have seen that inadequate vaccination exists in 10-30% of chickens that do not receive complete doses of vaccine. These birds will have poor or nil immune response. Thus it will allow the virus to replicate and perpetuate on the farm.
3. Periodically evaluate the manufacturing laboratories through chicken immunizations and check their antibodies levels. Choose the lab(s) who show a constant improvement in HA titers during a determined time.
4. Standardization of vaccines in terms of content of hemagglutinin per dose.
5. Spray the flock daily with disinfectant like ammonium quaternarium or citricus; moreover, disinfect the drinking water when the virus is present in the flock.
6. Use antibiotics joined with analgesic and expectorant. These help minimize the severity of the disease when the presence of opportunistic bacteria exists.
7. Increase the biosecurity measures.
8. Provide comfort zones to the chickens such as temperature, ventilation, and vital space.
9. Protect the flock against immunosuppressive diseases like infectious bursal disease, chicken anemia and mycotoxins, for example.

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COMPARISON OF REAL-TIME RT-PCR, CONVENTIONAL RT-PCR AND VIRUS ISOLATION ASSAYS FOR DETECTING AVIAN INFLUENZA VIRUS IN EXPERIMENTALLY INFECTED CHICKENS AND TURKEYS

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Real-time RT-PCR (RRT-PCR) assays, using 5’-nuclease oligoprobes that target the group specific matrix gene segment as well as subtype-specific H5 and H7 hemagglutinin gene segments (1), were compared with conventional, type-specific RT-PCR assays and virus isolation in embryonated chicken eggs. Groups of 10 chickens and turkeys were experimentally infected with 10 different strains of avian influenza virus listed below (Table 1).

Each bird was inoculated with 10^6 EID_{50} of virus by the oronasal route and serial oropharyngeal and cloacal swab specimens were collected and tested by the above assays.

Virus shedding, as determined by isolation in embryonated chicken eggs, could be first detected between two and five days post-inoculation (dpi) from oral and cloacal routes. In all cases, virus isolation was accomplished after a single passage in eggs. The

duration of virus shedding by both routes varied with virus strain but in general, cloacal shedding was more prolonged than oral shedding. For a number of the low pathogenic avian influenza virus strains tested, the duration of shedding was brief lasting only two to four days. In these experiments, cessation of viral shedding occurred just prior to the appearance of serum antibodies.

For all nucleic acid detection assays, RNA was extracted from swab specimens using the Qiagen RNeasy® Mini Kit in combination with a QIAvac24 vacuum manifold. Reaction mixes were set up using the Qiagen® One-Step RT-PCR kit. Conventional RT-PCR assays utilized H5 and H7 (2) specific primers which produced amplicons 863 bp and 1152 bp in size respectively. All RRT-PCR assays were carried out on the Cepheid Smart Cycler® platform using 25:l reaction volumes.

The diagnostic sensitivity and specificity of RRT-PCR and conventional RT-PCR assays were very similar to that observed with virus isolation in embryonated chicken eggs, as was the earliest and latest time points that virus could be detected post-inoculation. Given the rapidity and potential portability of RRT-PCR over that of virus isolation in eggs, results of the current study support the use of RRT-PCR as a front-line diagnostic tool in the management of avian influenza outbreaks.

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Table 1. Avian influenza virus strains used to compare nucleic acid detection and virus isolation assays.

Avian Influenza Virus Strain	Virulence
A/Turkey/Minnesota/3689-1551/1981 H5N2	LPAI
A/Chicken/Pennsylvania/1370/1/1983 H5N2	HPAI
A/Turkey/California/35621/1984 H5N3	LPAI
A/Turkey/Ontario/7732/1966 H5N9	HPAI
A/Turkey/Ontario/1963 H6N8	LPAI
A/Turkey/Ontario/18-2/2000 H7N1	LPAI
A/Chicken/Australia/3634/1992 H7N3	HPAI
A/Chicken/Victoria/32972/1985 H7N7	HPAI
A/Magpie Robin/China/28710/1993 H7N8	LPAI
A/Turkey/Minnesota/12877/1285/1981 H9N2	LPAI

BROAD-SPECTRUM PROTECTION AFTER THE USE OF AN INACTIVATED VACCINE CONTANING *SALMONELLA ENTERITIDIS* AND *SALMONELLA TYPHIMURIUM* STRAINS PRODUCED UNDER IRON-RESTRICTION

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ABSTRACT

The human health authorities and the poultry industry worldwide recognize the zoonotic implications of *Salmonella* infections in poultry. During the 1980s *Salmonella enteritidis* became of major importance as a predominant source for human salmonellosis, originating from poultry products. An eradication program to control not just *S. enteritidis* but all other *Salmonellae* associated with poultry may be unrealistic. For that reason vaccination continues to be a valuable tool that can be incorporated successfully in

Salmonella control program. The poster describes the benefits in terms of broader protection achieved after the use of an inactivated vaccine containing whole cell *S. enteritidis* PT4 and *S. typhimurium* DT104 strains produced under iron-restriction with aluminium hydroxide gel as an adjuvant.

In a first group of experiments SPF white leghorns were vaccinated intramuscularly with 0.5 ml of vaccine at four and six weeks of age. At eight weeks of age the birds were challenged orally with either *S. enteritidis* (serogroup D), *S. typhimurium* (serogroup B), *S. heidelberg* (serogroup B), *S. agona* (serogroup B), or *S. hadar* (serogroup C). Birds were inspected daily for clinical signs. Shedding of the challenge strains was assessed by cloacal swabs at different intervals up to 28 days post challenge. The *S. typhimurium* and *S. agona* data were derived from birds that had been in contact with seeder birds that had been infected as described above. The growth of *Salmonellae* on BGA-NAL-NOV plates or in selenite broth was expressed semi-quantitatively as heavy (>50 colonies), medium (1-5- colonies) or light (growth following enrichment in selenite broth only).

Vaccination of SPF birds resulted in a significant reduction of *Salmonella* shedding after challenge with *S. enteritidis*, *S. typhimurium, S. heidelberg* and *S. agona* under laboratory conditions. This reduction was observed not only for the number of birds shedding *Salmonella* but also in their levels of shedding. Vaccination did not reduce the shedding of *S. hadar* under the same experimental conditions.

In a second experiment the progeny of broiler breeders vaccinated at 12 and 16 weeks of age were orally challenged at one day of age with *S. typhimurium*. Weekly and until six weeks of age cloacal swabs were taken and cultivated directly on brilliant agar green (BGA) and pre-enriched in buffered peptone water and enriched in tetrathionate. The progeny of vaccinated breeders showed a gradual reduction in the excretion of *S. typhimurium* at the beginning, and thereafter an abrupt reduction in the last one to two weeks (slaughter age). This was true not only for the number of birds shedding the challenge strain but also for their level of shedding.

From the above it can be concluded that the combined *S. enteritidis* + *S. typhimurium* vaccine provides protection against challenge with strains belonging to the serogroups of the vaccine strains. The shedding of *Salmonella* after challenge is reduced (quantitatively as well as qualitatively) in the offspring of vaccinated birds.

(A full report of the study will be submitted for publication.)

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MOLECULAR IDENTIFICATION OF AVIAN RNA VIRUSES STORED ON FTA FILTER PAPER

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ABSTRACT

Collection, storage, and transport of pathogens from the field in foreign countries to centralized laboratories in the USA require an organized infrastructure involving compliances with strict regulations specified by the U.S Dept of Transportation, the International Civil Aviation, and the U.S. Dept of Agriculture. These requirements can be circumvented by the use of FTA filter papers which contain lyophilized chemicals that lyse prokaryotic and eukaryotic cells upon contact, thus inactivating possible infectious organisms (1). We have collected and stored a number of laboratory and clinical specimens on FTA papers and analyzed them for the presence of infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), and avian leukosis virus subgroup J (ALV-J). Inactivation of the viruses was demonstrated by their inability to grow in embryos or cell cultures. The stability of viral RNA or proviral DNA stored on the FTA cards was demonstrated by RT-PCR or PCR following prolonged storage and heat treatment, after which molecular identification by RFLP or sequencing analysis was still

feasible. IBV was readily detected in allantoic fluid stored at room temperature and at 41C for at least 15 days. RFLP or sequencing analysis of these samples clearly distinguished common variants like Arkansas, Connecticut and Massachusetts. Similarly, we detected IBDV in minced bursas stored on FTA for at least 13 days irrespective of the temperature of storage (-20C to 41C). RFLP analysis of some of these samples showed the pattern of Variant A, Variant E and Lukert. We also detected ALV-J as viral RNA or proviral DNA in infected fibroblastoid cell line cultures stored on FTA for at least 30 days and proviral DNA in liver and spleen tumors stamped on FTA and stored for 40-45 days.The use of the FTA/RT-PCR or FTA/PCR systems will reduce significantly the cost of transportation and perhaps increase the test sensitivity for the identification of avian pathogens from overseas.

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NEW FLOCKCHEK* AVIAN PNEUMOVIRUS ANTIBODY TEST KIT

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INTRODUCTION

The most common and prevalent types of avian pneumovirus (APV) are A and B, but there are concerns from epidemiologist of the potential spread of APV type C from wild flocks to the poultry industry. This phenomenon already occurs in the U.S. poultry market where APV type C is the most prevalent and distributed APV in turkey flocks in certain states (3).

For this reason, it is important for monitoring purposes to have a diagnostic test kit that is able to detect any of the potential APV strains in the commercial chicken or turkey industry.

These APV types can cause damage to the upper respiratory tract (trachea), such as lack of cilia movement and/or cilia loss-damage that may lead to upper respiratory clinical signs such as coughing, sneezing, swollen head, and more complicated respiratory problems (most probable situation in turkeys and broiler breeders). This stress on the cilia and upper respiratory tract can facilitate the multiplication of *E. coli* and other bacteria infections such as *Mycoplasmas*, *Bordetella* spp., etc. that lead to a respiratory syndrome called swollen head syndrome (SHS) (most probable situation in commercial layers and broilers). This co-infection factor has been demonstrated by Jones *et al.* (4). Also APV plays a role in the multiplication of infectious bronchitis virus (IBV) in the upper respiratory tract, which can facilitate an IBV vaccine rolling.

APV can colonize the reproductive tract affecting the small glands that excrete calcium and pigments during the egg formation in turkey breeders, broiler breeders, and laying chickens. The resulting lesions due to this colonization will cause a lack of pigmentation on the eggshell, increase in the percent of egg abnormalities (1,2), and increase the susceptibility of bacteria penetration through the eggshell because of the increase of eggshell cracks. The resulting drop in egg production will affect the whole production period.

Serological monitoring of avian pneumovirus vaccination and/or field infection of chickens and turkeys is a useful diagnostic tool. Because virus neutralization assay is more laborious and it lacks standardization, ELISA is used in more and more laboratories worldwide. IDEXX Laboratories has developed a new, user-friendly FlockChek* APV Ab Test Kit that specifically detects antibodies against the avian pneumovirus types A, B, and C. It is an indirect ELISA in order to be able to use it quantitatively when monitoring the APV status of vaccinated or non-vaccinated chicken and turkey flocks.

MATERIALS AND METHODS

The performance of the new FlockChek* APV Ab Test Kit has been evaluated testing chicken and turkey serum samples, experimentally and naturally infected and/or vaccinated with different strains of the APV virus in IDEXX's own laboratory, as well as at a few customer sites. The vaccines used were B type from two different vaccine companies, and A type from another vaccine manufacturer. The birds were primed with one or two live vaccines and boosted by killed vaccines according to the producer's vaccination routines. The sensitivity for the C type was monitored

by testing birds after experimental vaccination with an inactivated vaccine and also after a field infection outbreak with the APV C strain. The specificity of the test has been evaluated by testing SPF birds of different ages as well as poultry samples from the USA. All tests have been performed according to the package insert and the results were calculated as S/P values as well as titers.

RESULTS AND DISCUSION

The FlockChek* APV Ab Test Kit showed 98.3% specificity testing 1500 SPF chicken samples between the ages of 20-70 weeks from Germany. This is very good specificity in comparison to other commercial diagnostics currently on the market.

Vaccinated birds using A and B type vaccines started to show antibody titers following the first live vaccine, although seroconversion usually became complete only after the second vaccination. Birds injected intramuscularly with an experimental inactivated C vaccine from the U.S. showed seroconversion 11-14 days post-inoculation. Turkeys of different age groups showed a good rate of seroconversion following a C strain field infection.

There are several challenges of an APV serological test. It is important to achieve high specificity to be able to monitor SPF birds and negative field flocks. The quantitative application is helpful for monitoring vaccination programs and infectious status of commercial poultry flocks in a simple and economical way. The xChek software, developed for the IDEXX FlockChek product line, is a simple, user-friendly computer program for calculating and reporting antibody titers of the new FlockChek* APV Ab Test Kit.

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DIAGNOSIS ASSISTANCE SYSTEM FOR MAJOR CLINICAL POULTRY DISEASES

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The diagnosis is the crux for all medical problems. There are two ways of making a diagnosis in the practice of medicine. The role method is based on past experience (known as an educated guess) and depends upon the recognition of a syndrome or considering the accumulation of symptoms which is identical with one seen on a previous occasion. This method as experience is enlarged becomes reasonably accurate in many cases. However, its weakness lies in the fact that so few cases are identical and many are seen that are sufficiently atypical to make use of this method highly inaccurate. The second method is a diagnosis by reasoning and is based on rational summing up to clinical findings and progression by logical steps to a final diagnosis.

Logic process of elimination (Sherlok Holmes' method) is use in the database software, based on a systematic structure (order and relations) of abnormalities, symptoms, signs, productive damages and lesions in a pattern of diseases in order to cover all the "reasonable" disease possibilities.

As the diagnostic possibilities can be reduced to a small number, confirmation of the diagnosis by laboratory methods becomes so much easier because there are fewer examinations to be made, and confirmation by response to treatment is easier to assess. Most of the diseases of poultry can be identified on the farm, at least on a tentative basis. Laboratory findings can serve to confirm or deny a tentative identification. They should be employed as a second step to immediate efforts made on the farm to identify the problem.

The abnormalities, symptoms, signs, and lesions were taken from *Diseases of Poultry* by B.W. Calnek, 1991. Some differences between morbidity, diffusion, progression, mortality, and some "clue" diagnostic data came from by clinical experience.

This system aims to assist the diagnostician to have at glance those "clue", and not to take for granted

all those little details that make the differences between the diseases and enhancing the chances for accurate clinical diagnosis.

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GENOTYPE/ENVIRONMENT INTERACTION IN A COMMERCIAL LINE OF LAYING HENS

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SUMMARY

This work addresses the interactive effects of genotype and environment in flocks of laying hens coming from different breeder flocks varying in age and season of production from various farms located in the zone Altos de Jalisco, Mexico. The following were used as classifying variables: The genetic level of the flocks was considered to be the year of production; the seasonal observations were divided into spring/summer and fall/winter; and the age of the breeder hens was considered as young (Y) (hens at the peak of production) or old (O) (at the end of the production cycle).

MATERIALS AND METHODS

The information used in the present study consisted of production performance of one, two, and three year old flocks of W36 Hyline hens originating from different groups of breeder hens of varying age and season raised on farms in the Alto Jaliscan area.

The following variables were used for this study:

1) The genetic level as the production year (1, 2, or 3)
2) Hatch season as either spring/summer (SS) or fall/winter (FW)
3) Age of the breeder flock as either young (Y), consisting of hens at the peak of production; or old (O), comprising breeder flocks near the end of the lay cycle.

Covariables used:

1) Body weight of the breeder hens
2) Mean egg weight of the flock producing the commercial layers
3) Mean chick weight at hatch

Arcsin transformation (9) of percent mortality at 18 weeks of age and percent production of the commercial layers during weeks 20, 21, 22, 23, 24, 25, 26, 27, and 28 were used as dependent variables according to the following model:

$$\mathbf{Y_{ijkl} = \mu + G_i + E_j + C_k + I_{ij} + I_{ik} + B_1 X_1 + B_2 X_2 + B_3 X_3 + E_l}$$

Where:

$\mathbf{Y_{ijkl}}$ = percent mortality at 18 weeks in the commercial hens, or percent of production from weeks 20 to 28.

μ = a general mean

$\mathbf{G_i}$ = the genetic level of the breeders

$\mathbf{E_j}$ = the hatch season of the chicks

$\mathbf{C_k}$ = the age classification of the breeders

$\mathbf{I_{ij}}$ = the genotype-hatch season interaction

$\mathbf{I_{ik}}$ = the genotype-breeder age interaction

$\mathbf{X_1, X_2, X_3}$ = body weight, egg weight, and hatch weight respectively. These are used as covariables.

$\mathbf{B_1, B_2}$ **y** $\mathbf{B_3}$ = model adjustment parameters

$\mathbf{E_l}$ = random error value

We also evaluated the least squares estimator of the means and standard error of all dependent variables using GLM of SAS (version 6.12).

RESULTS AND DISCUSSION

Table 1 shows the least squares estimator of the means for percent mortality at 18 weeks and 20-28 week percent egg production for the W36 white hens. We noted that for percent mortality, the principal effects for genetic groups 1, 2, and 3 are 2.0, 2.8, and 1.6 respectively, denoting significant differences at the genetic levels 2 and 3 ($p<0.05$). This could be attributed more to pathologic problems during the growing stage of genetic level 2 flocks, since they

experienced higher mortality, than to genetic difference between groups. No significant difference ($p<0.01$) was seen in seasonal percent mortality (SS = 2.5% and FW = 1.8%), nor was there significant difference in mortality between offspring from Y or O breeder hens (Y pullets exhibiting 3.2% mortality and O pullets, 1.1%).

The principle effects for the beginning of lay at 20 weeks of age, expressed in percentages, is shown in Table 1 where we note that genetic levels 1 and 2 begin lay with a 2.2% production rate while level 3 initiated lay at 11.8%. This difference is significant (p<0.05) through week 23, where group 3 reached 64.5%; group 2, 48.5%; and group 1, 51.2%. From 24 to 28 weeks, however, there was no significant difference in percent egg production between genetic levels. At week 28 egg production in group 1 was 89.4%, group 2 was 90.5%, and group 3 reached 89.5%.

Regarding the seasonal effects on the initiation of egg production in the commercial hens, the pattern is similar to that seen in the genetic groups, as the FW hens initiated lay at 1.6% while SS hens were 9.1%. There was a significant difference (p<0.05) until week 23, where FW hens reached 43.9% and the SS hens attained 65.6% egg production. Beginning at week 24, and extending through week 28, there was no detectable difference (p>0.01) between groups. At 28 weeks, the FW hens reached 89.0% production and the SS hens laid at 90.6%.

No significant difference was found between layers hatched from either Y or O breeder hens. The Y layers began lay at 4.1%, reaching 90.8% at week 28. Layers from O breeder hens initiated lay at 6.7% and at week 28 reached 88.9% egg production.

Table 2 shows the mortality percentages expressed as the least squares estimator of the means, showing the effect of the interaction between the genetic level and season. Of the six combinations, 2, 5, and 6 show statistically significantly difference from combinations 1, 3, and 4, with percent mortality of 1.2, 1.6 and 1.7 compared to 2.8, 3 and 2.5 respectively. Combination 2 represents genetic level 1 and season SS; combination 5 is genetic level 3 and season FW; and combination 6 is genetic level 3 and season SS. The significance of these is not only biological, but also economic, when one percentage point may be the difference between profit or loss.

From the previous description it can be inferred that the combination of genetic level 3 and age O show tendency to start laying at higher rates in the first four weeks of production, but after the fifth week there are no significant differences among the six combinations. Therefore, there is a need to determine the impact of genetic/environmental interactions on the complete laying cycle and egg mass to be able to select those flocks that will have the best performance for a given environment.

CONCLUSIONS

Gentic/environmental interactive effects are noted between genetic level and season, for 18-week mortality, and for the beginning of the lay cycle. Also, genetic/environmental effects are found between gentic level and age of breeder hens referring to 20 to 23-week percent egg production ($p < 0.05$).

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Table 1. Least squares estimator of the means for percent mortality and 20 to 28 week egg production in Hy-Line laying hens.

	Genetic level			Season		Age	
	1	2	3	FW	SS	Y	O
Mort. 18 Weeks	2.0[a]	2.8[ab]	1.6[ac]	2.5[a]	1.8[a]	3.2[a]	1.1[a]
Prod. 20 Weeks	2.2[a]	2.2[a]	11.8[b]	1.6[a]	9.1[b]	4.1[a]	6.7[a]
Prod. 21 Weeks	9.4[a]	9.9[a]	26.8[b]	6.2[a]	24.5[b]	9.4[a]	21.3[a]
Prod. 22 Weeks	26.3[a]	25.9[a]	46.4[b]	19.3[a]	46.5[b]	8.0[a]	57.8[a]
Prod. 23 Weeks	51.2[a]	48.5[a]	64.5[b]	43.9[a]	65.6[b]	1.0[a]	100.0[a]
Prod. 24 Weeks	71.8[a]	67.8[a]	78.8[a]	67.5[a]	78.1[a]	20.6[a]	100.0[a]
Prod. 25 Weeks	80.7[a]	80.0[a]	79.0[a]	75.7[a]	84.1[a]	57.7[a]	100.0[a]
Prod. 26 Weeks	86.4[a]	86.7[a]	85.1[a]	82.6[a]	89.5[a]	61.7[a]	100.0[a]
Prod. 27 Weeks	88.8[a]	90.4[a]	89.7[a]	87.7[a]	91.6[a]	63.6[a]	100.0[a]
Prod. 28 Weeks	89.4[a]	90.5[a]	89.5[a]	89.0[a]	90.6[a]	90.8[a]	88.9[a]

Note: Means within a column with no common superscript differ significantly ($p<0.05$).

Table 2. Percent mortality at 18 weeks, expressed as the least squares estimator of the means, showing genotype/seasonal effects in Hy-line laying hens.

Genetic Level	Season		Age	
	FW	SS	Y	O
1	2.8[a]	1.2[bc]	3.0[a]	1.0[a]
2	3.0[a]	2.5[a]	3.9[a]	1.7[a]
3	1.6[ac]	1.7[bc]	2.7[a]	0.6[a]

Note: Means within a column with no common superscript differ significantly ($p<0.05$).

FIRST FUNCTIONAL ASSESSMENT OF A CHICKEN CASPASE

Y. Song

We have cloned and expressed chicken caspase 8. Transfection of this plasmid construct into both avian and mammalian cells resulted in apoptosis as measured by several assays. These data will be presented. This is the first functional assessment of a chicken caspase and opens the door for further exploration of apoptosis in avian disease pathogenesis.

EVALUATION OF THE EFFICACY OF VACCINATION AGAINST NEWCASTLE DISEASE IN CANARY BIRDS (*SERINUS CANARIUS*)

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ABSTRACT

In 2000 Italy was affected by an epidemic of Newcastle disease (ND) (1). Among the restrictive measures to control the spread of the disease was an increase of compulsory vaccination program for all susceptible species, included pet birds. The aim of this study was to test the susceptibility of canary to high pathogen NDV and the efficacy of a vaccination program specific for this species.

A vaccination program with commercially available live and inactivated vaccines was performed in order to have an effective and enduring immunity able to protect the birds from the clinical disease and to decrease the viral shedding. Fifteen birds were vaccinated with live attenuated vaccine (BIO-VAC® B1, Fatro S.p.A.) admistrated by spray at 1 and 21 days of age and then by subcutaneous route in the neck skin (0,02 ml/10 g) with inactivated oil emulsion vaccine (OL-VAC®: Fatro S.p.A.) at 35 and 56 days of age. Seven birds were left as unvaccinated control. The

efficacy of the vaccination was tested by using a 100 µl of a viral suspension with $10^{8,5}$ EID_{50} of high pathogen NDV strain administrated oro-nasally in both groups of birds five weeks after the last vaccination.

The challenge confirmed the low susceptibility of the canary to NDV infection (2,3): 2/7 unvaccinated birds did not show any clinical signs in spite of the high infectious dose utilized, and no viral shedding was found in feces of the vaccinated and control group. Clinical signs were observed in 5/7 birds of control group. They showed severe depression and nervous signs prior to death. The efficacy of the vaccination program tested was very high and no vaccinated canary showed clinical signs of Newcastle disease after challenge. The difference in clinical signs between the vaccinated group and the control group was statistically significant ($p<0.01$).

The clinical signs, the results of virological investigations, and the histopathologic lesions of unvaccinated dead birds show that ND in canary could have characteristics of marked neurotropism. Further studies need to determine if it depends either on challenge virus or on characteristic of the species.

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BIOCHEMICAL AND 16S rRNA SEQUENCING CHARACTERIZATION OF *PASTEURELLA HAEMOLYTICA*-LIKE ORGANISMS IN COMMERCIAL LAYER FLOCKS IN MEXICO

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INTRODUCTION

Several consistent reports involving avian *Pasteurella haemolytica* with lesions in chickens e.g., salpingitis, septicemic conditions and peritonitis were reported. *Pasteurella haemolytica*-like organisms from commercial chicken flocks were identified from different regions of Mexico (1998-2003). In this study we had analyzed 209 *P. haemolytica*-like isolates involving 41 clinical cases. According with the phenotypic analysis proposed by Christensen *et al*. (2003) five biovars (3, 12, 17, 22 and 24) were identified and 8 different biochemical patterns were observed. Other five strains were identified as *Gallibacterium* based on 16srRNA sequencing. *Gallibacterium* was recently established as a new genus within the family *Pasteurellaceae*(1). Bacteria belonging to this genus have previously been reported as *Pasteurella anatis*, avian *Pasteurella haemolytica*-like organisms or *Actinobacillus salpingitidis*.

MATERIAL AND METHODS

Isolates (209) were obtained from breeders, broilers, and layers. Bacteria were isolated from trachea and palatine cleft and some of them from ovary and heart from birds with respiratory or reproductive problems in several regions of Mexico. Bacterial isolates were propagated from preserved stocks in brain heart infusion broth (BHI) supplemented with 15% glycerol and stored at –70ºC. Biochemical identification and 16s rRNA sequencing were done according with Christensen *et al*. (1).

RESULTS

Pasteurella haemolytica-like was isolated mainly in layers with drop in egg production associated with clinical signs and lesions such as anorexia, peritonitis, hepatitis, severe nephritis, dehydration, and arthritis. Decrease in egg production was in the range from 4-20%. We identified five different biovars 3, 12, 17, 22 and 24 corresponding to 24 biovars reported by Christensen *et al*. (1). One isolate of biovar 3 recovered from palatine cleft, in broilers of four weeks of age in

Veracruz State. Six isolates of biovar 12 recovered from spleen, ovary and liver, in layers of 26 weeks of age in Puebla, Jalisco and Yucatán states. One isolate of biovar 17 recovered from palatine cleft, in layers of 23 weeks of age from Veracruz State. Three isolates of biovar 22 recovered from palatine cleft, in layers of 26 weeks of age in Puebla State. One isolate of biovar 24 recovered from palatine cleft in breeders in Veracruz state. Other five strains non-identified in the Christensen biovar pattern were identified as *Gallibacterium anatis* by the analysis of the 16S rRNA gene sequences.

CONCLUSIONS

The results from this study and recent investigations strongly suggest that *Gallibacterium* is an underestimated cause of disease in commercial chickens. Additionally, recent development of molecular tools and an infection model has paved the way for detailed analysis of the impact of specific bacterial and host related factors employed in the pathogenesis of *Gallibacterium* infection in chickens. Outbreaks in Mexican poultry farms have caused considerable losses due to *Gallibacterium* infections and have also been reported from a number of disease outbreaks in poultry in other countries (2, 3).

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VIRUSES ASSOCIATED WITH POULT ENTERITIS IN TURKEYS GROWN IN CALIFORNIA 1993-2003

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Poult enteritis is one of the most common diseases seen in young turkey flocks. Clinical signs include loose droppings and increased mortality. Grossly, the small intestines have pale serosa, segmental dilation, and watery contents. Since 1993, more than 1800 cases of suspected poult enteritis have been submitted for examination by negative stain electron microscopy; this has involved more than 2400 result entries of which at least 1500 were positive for viruses. Viruses have been identified in poults as young as three days and up to nine weeks of age. The most commonly found viruses are rotavirus, and small round viruses ranging in size from 15-30 nm, either alone or in combination. Reovirus, birnavirus, and adenovirus have also been detected. There has been no evidence to suggest the presence of coronaviruses.