PROCEEDINGS OF THE FIFTY-SIXTH WESTERN POULTRY DISEASE CONFERENCE

March 26-29, 2007 Las Vegas, Nevada

THE 56th WESTERN POULTRY DISEASE CONFERENCE DEDICATION

R. Keith McMillan

R. Keith McMillan was born and raised in Saskatchewan and was a proud Canadian who spent almost his entire career servicing the poultry industry in Western Canada. Keith obtained his DVM from the University of Saskatchewan, Western College of Veterinary Medicine in 1973. He received his MSc. in poultry medicine and pathology in 1982 from the University of Saskatchewan, Western College of Veterinary Medicine. In 1992 he received his Board Certification as a Diplomate in the American College of Poultry Veterinarians. In 2003 Dr. McMillan received his on farm audit certificate for On-Farm Food Safety Programs in Alberta.

From 1973 to 1979 Keith worked as a "multi-species" veterinarian in Saskatchewan, concentrating on dairy and swine commercial livestock production. From 1979 to 1983 Keith worked as the Extension Veterinarian at the University of Saskatchewan, Western College of Veterinary Medicine. From 1983 to 1989 Keith operated a private veterinary practice specializing in poultry medicine. Keith held the position of Vice President of Quality Assurance and Veterinary Affairs at Lilydale Foods from 1989 to 2003.

Dr. McMillan passed away June 2, 2006. Keith was a friend and colleague to many, and his professional and family values were outstanding. His wife Julie has the same qualities and together they lead by example.

56th WPDC SPECIAL RECOGNITION AWARD

MASAKAZU MATSUMOTO

Masakazu Matsumoto was born October 28, 1941 in Tokyo, Japan. He earned his B.S. and D.V.M. degrees at the University of Hokkaido, Sapporo, Japan and subsequently received his M.S. in microbiology from the University of Hawaii in 1966. After Hawaii, he was accepted at the University of California, Davis and earned a Ph.D. in 1972 in comparative pathology. His major professor at UCD was Dick Yamamoto. After a one-year immunology post-doctoral stint at Purdue University, Dr. Matsumoto worked at Plum Island Animal Disease Center as a microbiologist. In 1975 he accepted a position with Oregon State University, Corvallis Oregon, where he retired as a full professor in 1999. He diagnosed the first case of infectious coryza in Oregon.

Dr. Matsumoto met his wife, Nancy, at a dance party held on UCD campus. They were married in 1971. Other members of the family are a son, Paul; daughter, Mari; and granddaughter, Elina.

Because of a prior commitment, Dr. Matsumoto knew he would be unable to attend the WPDC in Las Vegas and in lieu sent his regards and the following comments:

> I am honored to receive this award, and I am sorry for not being here because of prior commitments. I was literally professionally born and raised by WPDC, and as such, would like to thank everybody in WPDC for the kind support and patience through so many years.
>
> In the fall of 1967 I was hired as a researcher under Dr. Yamamoto. At WPDC held in March of 1968, I presented my first research paper. It was held in the auditorium of the newly constructed chemistry building on UCD campus. When I started my presentation, a gentleman in the first row interrupted me practically after each sentence I spoke, saying, "You actually mean such and such," or "Did you actually prepare each batch from a single or multiple sources?", etc. My talk was supposed to last 10 minutes, but it took over 30 minutes. During the following break, some people approached me and asked, "How can you stand $#@!* Julius?" The gentleman was Julius Fabricant of Cornell. However, I did not think that he was rude, but that he was extremely kind to clarify some points that strengthened my presentation. I thanked Julius and shook hands with him. Following this encounter, we became good friends and talked at numerous occasions. Through this first experience, I understood the nature and purpose of WPDC, and attending WPDC became an essential part of my professional life.
>
> Through WPDC I developed many professional friends, whose help was indispensable to solve many local problems in avian medicine. I would like to mention the names of all of these friends, but obviously I can't. However, I must recognize three people who greatly helped me during my formative years in avian disease research: Richard Yamamoto, who constantly encouraged my study and research; the late Henry Adler, who emphasized that creating new ideas is essential in research; and Rosy (A. Rosenwald), who alerted me many times to direct the research towards practical terms.
>
> I hope that WPDC will remain as a valuable communication place for researchers and diagnosticians in avian medicine for many years to come. Again, I want to thank all of you for this honor.

It is an honor for all of us to recognize Dr. Masakazu Matsumoto as our 2007 award recipient!

SPECIAL ACKNOWLEDGMENTS

The Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and to help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. More than 40 organizations, companies and individuals have 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 give a special acknowledgement two supporters at the Benefactor level. They are the **American Association of Avian Pathologists** and **Merial, 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 sincerely thank them and their representatives.

Dr. Bruce Charlton would like to thank Dr. Rich Chin for going beyond his role as Secretary-Treasurer in assisting the Program Chair and the entire Executive Committee. Without his input, deadlines would be missed, individuals would be forgotten, consistency would not exist, and the excellent caliber of the conference would cease. A special thank you also needs to be given to Pam Wyckoff for her assistance in corresponding to all presenters and in keeping the program chair on task. Thanks to the Executive Committee for their input. I truly appreciate it and apologize if I offended anyone by not following their suggestions. I would also like to thank all the invited speakers – Dr. Mark Bland, Dr. Erick Gingerich, Dr. Fred Hoerr, Dr. Jose Linares, and Dr. Linnea Newman. It has been a delightful educational experience to have listened to each of you in the past and have you share some of your experiences at our regional meeting. Finally, I would like to thank all presenters for sharing with the audience their particular interests. The duties of the Program Chair are minor compared to the opportunity of working with all of our colleagues in the poultry field.

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 Ekaterina Stone of the Fresno branch of the California Animal Health and Food Safety Laboratory System (CAHFS) 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 Ms. Teresa Brown, Ms. Jennifer Thayer, and Ms. Katrina Damilano 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, Staff Assistant III of the Utah State University Turkey Research Facility, for her seemingly endless hours of proofreading and formatting the Proceedings for publication. We express our gratitude to all authors who submitted manuscripts. A special thanks goes to Utah State University Cooperative Extension for once again reproducing the CD-ROM and donating the cost of reproduction for this year's meeting. We again acknowledge and thank Ominpress (Madison, WI) for the handling and printing of this year's Proceedings. Once again, we acknowledge Bruce Patrick (Graphic Communications, Brigham Young University) for the cover design of the printed proceedings and Dr. Rocio Crespo (CAHFS-Fresno) for original design of the CD label.

56th WPDC CONTRIBUTORS LIST

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56th WPDC PROCEEDINGS

The Proceedings of the 56th Western Poultry Disease Conference are not refereed, but are presented as a service and a source of information to those attending the conference and to others who wish to gain some insight as to the information presented. Copies of the Proceedings are available in either hardcopy or electronic (CD) formats.

Copies of these Proceedings are available from: Dr. R. P. Chin
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Five-year Compilation (2000–2006) Proceedings of the WPDC. This CD contains the printed proceedings of the 51st through the 55th Western Poultry Disease Conferences. Copies can be purchased from the WPDC Secretary-Treasurer.

WESTERN POULTRY DISEASE CONFERENCE (WPDC) HISTORY

	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
1st WPDC – 1952		A. S. Rosenwald		
2nd WPDC – 1953	P. D. DeLay	A. S. Rosenwald		
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11th WPDC – 1962	D. V. Zander	R. V. Lewis		
12th WPDC – 1963	R. V. Lewis	Walter H. Hughes		
13th WPDC – 1964	W. H. Hughes	Bryan Mayeda		
14th WPDC – 1965	B. Mayeda	R. Yamamoto		
15th WPDC – 1966	R. Yamamoto	David S. Clark		
		1st sign of Contributors		
16th WPDC – 1967	D. S. Clark	Roscoe Balch		
17th WPDC – 1968	R. Balch	Richard McCapes		
18th WPDC – 1969	R. McCapes	Dean C. Young		
19th WPDC – 1970	D. C. Young	W. J. Mathey		
4th Poultry Health Sym. (PHS)		**1st combined WPDC & PHS, 1st listing of distinguished members**		
20th WPDC – 1971	W. J. Mathey	Ramsay Burdett		
5th PHS				
21st WPDC – 1972	R. Burdett	Marion Hammarlund		
6th PHS				
22nd WPDC – 1973	M. Hammarlund	G. W. Peterson		
7th PHS				
23rd WPDC – 1974	G. W. Peterson	Craig Riddell		
8th PHS				
24th WPDC – 1975	C. Riddell	Ralph Cooper		
9th PHS				
25th WPDC – 1976	R. Cooper	Gabriel Galvan		
10th PHS				
26th WPDC – 1977	G. Galvan	Don H. Helfer	Hector Bravo	
11th PHS				
27th WPDC – 1978	D. H. Helfer	Art Bickford		
12th PHS				
28th WPDC – 1979	A. Bickford	J. W. Dunsing		
13th PHS				
29th WPDC – 1980	J. W. Dunsing (WPDC)	G. Yan Ghazikhanian	P. P. Levine	
14th PHS				
5th ANECA	Angel Mosqueda T. (ANECA)			
30th WPDC – 1981	G. Y. Ghazikhanian	Mahesh Kumar		
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31st WPDC – 1982	M. Kumar	Robert Schock		
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33rd WPDC – 1984	G. B. E. West	Gregg J. Cutler		
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36th WPDC – 1987	D. A. McMartin	Marcus M. Jensen		
37th WPDC – 1988	M. M. Jensen	Barry Kelly	A. S. Rosenwald	
38th WPDC – 1989	B. Kelly	Masakazu Matsumoto		Louise Williams
39TH WPDC – 1990	M. Matsumoto	Jeanne M. Smith		Dean Young
40th WPDC – 1991	J. M. Smith (WPDC)	Richard P. Chin (WPDC)	A. S. Rosenwald	
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41st WPDC – 1992	R. P. Chin	Rocky J. Terry	Marcus Jensen	Henry E. Adler (posthumous) R. A. Bankowski C. E. Whiteman
42nd WPDC – 1993	R. J. Terry	A. S. Dhillon	W. W. Sadler	Royal A. Bagley
43rd WPDC – 1994	A. S. Dhillon	Hugo A. Medina		G. B. E. West
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45th WPDC – 1996	D. D. Frame (WPDC)	Mark Bland (WPDC)	Don Zander (WPDC)	Pedro Villegas
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48th WPDC – 1999	H. L. Shivaprasad	R. Keith McMillan		
49th WPDC – 2000	R. K. McMillan	Patricia Wakenell	R. P. Chin	Ralph Cooper Robert Tarbell
50th WPDC – 2001	P. Wakenell	Ken Takeshita		Don Bell Art Bickford
51st WPDC – 2002	K. Takeshita	Barbara Daft	Hiram Lasher	Bachoco S.A. de C.V. Productos Toledano S.A.
52nd WPDC – 2003	B. Daft	David H. Willoughby		Roland C. Hartman
53rd WPDC – 2004	D. H. Willoughby	Joan Schrader		G. Yan Ghazikhanian
54th WPDC – 2005	J. Schrader	Stewart J. Ritchie	W.D. Woodward	R. Keith McMillan
55th WPDC – 2006	S. J. Ritchie	Peter R. Woolcock		M. Hammarlund
56th WPDC – 2007	P.R. Woolcock	Bruce Charlton	R. Keith McMillan	M. Matsumoto
57th WPDC – 2008	B. Charlton	Rocio Crespo		

MINUTES OF THE 55TH WPDC ANNUAL BUSINESS MEETING

In the absence of President Ritchie, Secretary-Treasurer Chin called the meeting to order on Tuesday, 7th March 2006, at 4:45 PM, at the Holiday Inn Capitol Plaza, Sacramento, CA. There were about 40 people in attendance.

APPROVAL OF 54th WPDC BUSINESS MEETING MINUTES

The minutes from the 54th WPDC business meeting were reviewed and a motion was carried to approve them as printed in the Proceedings of the 55th 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, Inc. He also thanked all the contributors for their generous donations. Secretary-Treasurer Chin acknowledged the efforts of the current WPDC officers for their work and participation in the organization of this year's meeting.

REPORT OF THE SECRETARY-TREASURER

Dr. R.P. Chin presented the Secretary-Treasurer report. There were 256 registrants for the 54th WPDC held at the Fairmont Hotel Vancouver, BC, Canada, April 25-27, 2005. Contributions for the 54th WPDC were $36,742, with a total income of $89,961. There were expenses of $77,965 for WPDC for the meeting, resulting in a net gain of $11,996. The current balance in the WPDC account was $57,820. Dr. Stewart Ritchie was congratulated for the outstanding support he raised for the meeting in Vancouver. More than $10,000 came from organizations (private and governmental) solely within Canada.

With this year's meeting going back to Sacramento, Dr. Chin was again unsure as to the number of registrants, and thus estimated another potential loss of approximately $2900. Estimated expenses for this year are approximately $65,000.

Once again, Dr. Chin stated that WPDC needs to look at ways to increase our registration numbers and contributions to break even for future conferences, which is why the WPDC will be held next year (2007) in Las Vegas, NV.

REPORT OF THE PROCEEDINGS EDITOR

Dr. D. Frame presented the Proceedings Editor report. There were 63 papers and a total of 105 pages in this year's proceedings. Publication of the Proceedings went rather smoothly this year and acknowledged the excellent assistance of Ms. Sherry Nielson. Dr. Frame reported that WPDC has partnered with Utah State University Cooperative Extension in the replication of the CD. USU Extension is very supportive of WPDC and donated the cost of production of the CD label and replication. Dr. Schrader recommended that the current President of WPDC write a letter to USU thanking them for their contribution and support. One challenge was trying to work with authors who submitted color photos or excessive graphs and figures. Dr. Frame recommends that photos should not be submitted. In addition, authors need to adhere to the guidelines in "Instructions to Authors" that would make editing and formatting more efficient. This year, for some reason, more manuscripts were sent to the Program Chair rather than to the Proceedings Editor. Again, Omnipress produced the Proceedings hard copy at an approximate cost of $4.88 per book.

OLD BUSINESS

None discussed.

NEW BUSINESS

Secretary-Treasurer Chin reported that the WPDC Executive Committee nominated Dr. Rocio Crespo for Program Chair-elect of the 57th WPDC in 2008, which will be a joint meeting with ANECA tentatively scheduled in Puerto Vallarta. There were no other nominations and Dr. Crespo was elected unanimously as program chair-elect. Secretary-Treasurer Chin nominated the following officers for 2006-2007:

Program Chair: Dr. Bruce Charlton
President: Dr. Peter Woolcock
Local Arrangement Coordinator: Dr. James Andreasen
Contributions Chair: Dr. Yan Ghazikhanian
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Rich Chin
Program Chair-elect: Dr. Rocio Crespo

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

The WPDC Executive Committee recommended that the 56th WPDC be held in Las Vegas, NV. Dr. Andreasen found a suitable venue at the Riviera Hotel & Casino, the week of March 26-30, 2007. The other possibility would be to return the Sacramento. A vote was taken and Las Vegas, NV for the 56th WPDC was approved. It was also announced that ANECA would like to have another joint meeting in Mexico, either in Puerto Vallarta or Guadalajara. This was voted upon and approved to join ANECA in 2008 for a joint meeting in Mexico and recommended Puerto Vallarta as the location.

Dr. Chin discussed having the WPDC with the Western Veterinary Conference every year in Las Vegas as a way to improve attendance. There were pros and cons for this and it was decided more investigation was needed.

Dr. Gregg Cutler brought up the issue of continuing education (CE) credits for WPDC. He said that there was a discrepancy between the number of hours of talks and what was being offered by the School of Veterinary Medicine at the University of California, Davis. Dr. Chin said that he works with Dr. Klingborg, Associate Dean, Veterinary Extension & Public Programs, on this. Each year, the requirements have been changing, and it is getting more difficult to get full credit for all the talks for CE credits for California veterinarians. It was proposed that WPDC have a CE Coordinator to work with UC Davis and Dr. Cutler agreed to do this.

Secretary-Treasurer Chin passed the presidency to Dr. Peter Woolcock who thanked those involved in the organization of the meeting. President Woolcock adjourned the meeting at approximately 5:30 PM.

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Note: Both the oral and poster presentations of the 56th WPDC are listed below in alphabetical arrangement by presenter. Authors and titles of all papers scheduled for presentation at the conference are listed.

PROCEEDINGS OF THE FIFTY-SIXTH WESTERN POULTRY DISEASE CONFERENCE

CASE REPORTS FROM ALABAMA

Frederic J. Hoerr

Thompson Bishop Sparks State Diagnostic Laboratory, P.O. Box 2209, 890 Simms Road,
Auburn, Alabama 36831-2209

CASE REPORTS

Gangrenous dermatitis. Broiler chickens developed increased mortality at 31 days of age. Mortality at 33 days of age when birds were submitted to the diagnostic laboratory was 560 of 20,000. Six birds had cutaneous lesions typical of gangrenous dermatitis (GD), and the bursae of Fabricius and thymus lobes had severe atrophy. PCR detected chicken anemia virus (CAV) in spleen, and real-time RT-PCR detected avian paramyxovirus type 1 in a pharyngeal swab (matrix primers positive, fusion primers negative).

Histologic examination of skin showed acute necrotizing dermatitis with intralesional large bacterial rods, consistent with GD caused by *Clostridium* spp. Bursa and thymus were evaluated for lymphocyte depletion, respectively, with 1=normal, 2=mild depletion, 3=moderate depletion, and 4=marked to severe depletion. For this case, bursa scores were 4,4,4,4, and 4, and for thymus, 4, 4, 4, 4 and 4, consistent with the onset of subclinical infectious bursal disease (IBD) and chicken infectious anemia (CIA) before 33 days of age, likely following the decline of maternal immunity, respectively.

Infectious bursal disease and CIA are risk factors for GD (7), although CIA is described primarily as a risk factor for blue wing disease of chickens 14 days of age or younger and lacking maternal immunity to chick anemia virus (CAV).

A retrospective study of GD cases seen at the Alabama state diagnostic laboratories from 1995-2006 revealed that marked to severe lymphocyte depletion from bursa and thymus usually occur with the onset of GD in broilers 14 days and older, with an average age of 39 days. PCR detection of CAV is common in these broilers (additional information to be published in *Avian Diseases*).

Long bone deformities in broilers. Long bone deformities affected broilers of all ages in a production complex. Splay-legged chicks sitting on reddened hocks were identified from the pipping embryo stage through all stages of chick processing in the hatchery. In the field, long bone deformities required culling through processing age. Legs from affected broilers of all ages were submitted for histologic examination.

Chicks with red hocks had histologic lesions of congested vessels and stasis of blood generally affecting the skin and connective tissues of the hock; hemorrhage occurred in some joint spaces. In birds from the field, the epidermis over the hock had erosion and ulceration with congestion, hemorrhage, and heterophil exudation affecting dermal connective tissue and peritendonal soft tissues, consistent with pressure trauma from sitting in sternal recumbency.

Three basic histologic lesions occurred in diaphyseal bone. The first involved wrinkling or bending of the most proximal or distal aspects of the diaphyseal bone with congestion and/or necrosis of the periosteum. This lesion occurred in chicks in the hatchery and in older broilers from that same hatchery. The second lesion was a wavy deviation in the diaphyseal bone, usually presenting as a slight indentation, suggestive of bending from biomechanical stress. The third lesion was either aseptic necrosis or obvious microscopic fracture of diaphyseal bone. In most cases, this small fracture occurred on one side of the bone. In a few cases, the fracture was visible on sides of the bone in the plane of section. In one particular leg there was a telescopic fracture of the shaft of the femur, suggestive of a high-impact, load-bearing stress on the end of the bone. Lesions occurred in the proximal and distal femur, more in the proximal than distal tibia, and in the proximal tibiotarsus.

The periosteum had focal congestion and hemorrhage usually adjacent to deviation of diaphyseal bone. In a few bones, pyknotic cells in the periosteum occurred with or without vascular events, consistent with focal necrosis or hypoxia of the periosteum.

Matching the histologic findings with field observations, two issues emerged. The red hocks in pipping and hatching embryos were suggestive of physiologic stress during hatching. Bending, microfractures, and periosteal lesions in the diaphyseal bones were consistent with impact trauma either from the side or directly along the long axis of the bone, and were considered to be directly related to long bone deformities. Resolution of the long bone deformity problem focused on the mechanical chick handling equipment in the hatchery, and addressing the environmental issues affecting hatch physiology.

Suspected zinc phosphide intoxication. A flock of 20,000 broilers developed mortality of 900 chicks greater than expected at four days of age. Chicks continued to eat and drink, but developed ruffled

feathers and died. Zinc phosphide rodenticide had been placed in the house when the contract grower was unable to obtain the rodenticide specified by the broiler program. The chicks were observed to have access to the rodenticide.

At necropsy, liver lesions were variable swelling, hemorrhage, and yellow discoloration. The proventriculus and gizzard content had a chemical odor, and the gizzard mucosa had hemorrhages and fissured koilin lining. Histologic lesions in liver were coarse vacuolar change in hepatocytes, hemorrhage, and hepatocyte necrosis of variable severity. Gizzard had necrosis and ulceration of the mucosa and heterophil exudation associated with koilin fusion defects. Lungs were congested.

Proventriculus was collected but lost during the laboratory investigation, thus precluding analysis for zinc phosphide. Neither arsenic (liver) nor lead (kidney) was detected at a significant concentration.

The history and lesions were consistent with zinc phosphide intoxication that was not confirmed by analysis. Zinc phosphide is a rodenticide with a garlic odor that is attractive to rodents, and has been in use for nearly 100 years. Gallinaceous birds are sensitive to intoxication. The action of gastric acid on zinc phosphide liberates toxic phosphine gas. Due to the rapid change to phosphine gas in the presence of gastric acid, zinc phosphide analysis is subject to misleading results (3, 5). The gas has an odor of acetylene, and is irritating and causes gastritis. Lesions at necropsy include visceral congestion, gastritis, and yellow liver.

Suspected organic arsenic intoxication in heavy breeder pullets. Broiler breeder pullets, six to seven weeks old, had increased culling and mortality. At necropsy, livers had multifocal green discoloration. Histologic examination of liver revealed marked dilation of intrahepatic bile ducts with villous folding of bile duct epithelium, suggestive of collapse following postmortem draining of bile. Some large bile ducts had ulceration of the mucosa accompanied by a coagulum of inspissated bile and a few cells. Adjacent to large bile ducts and multifocal throughout the liver were foci of fibrinoid and coagulation necrosis of hepatocytes. Heterophils attended acute lesions. In chronic lesions, large granulomas comprised a central zone of necrosis surrounded by giant cell macrophages and a peripheral zone of fibrosis. Some granulomas contained intralesional bacteria. Moderate to severe proliferation of bile ducts was associated with heterophilic and mononuclear cell inflammation in and around proliferating bile ducts. Additional livers submitted two weeks later showed the lesions above with prominent casts of inspissated bile and bile duct proliferation.

The lesions demonstrated marked to severe insult to the biliary system, resulting in either occlusion or stasis of bile flow. These broiler breeders were administered 4-nitrophenylasonic acid (Histostat 50, nitarsone) in the feed and simultaneous 3-nitro-4-hydroxy phenylarsonic acid in the water to prevent histomoniasis. Intoxication by 3-nitro-4-hydroxy phenylarsonic acid (Roxarsone, 3-Nitro) caused ulcerative cholecystitis in broilers (6) and turkeys (2). A review of dosages, drug inventories, and proportioner settings revealed no obvious errors. Fresh tissues were not collected for arsenic analysis, and analysis was not attempted on formalin-fixed tissue. The product data sheet for Histostat 50 (www.alpharma.com/ahd/pdf/Histo.PDF) emphasizes the importance of adequate drinking water to avoid intoxication. Some pullet flocks on this drug program developed hepatotoxicity and others did not; drinking water availability or palatability may have been a differentiating factor. There was some evidence of an ascending biliary bacterial infection; however, this was not a prominent feature of the acute lesion.

Suspected vitamin D_3 intoxication in broilers. Broilers in several flocks in a complex were developing rickets between one and three weeks of age during the hottest period of the summer. Heat and possible Ca:P balance in the feed were suspected as the cause. The flock of interest was 17 days old when feed was delivered in the morning, soon followed by broilers becoming recumbent primarily in the front half of the house. The service representative added vitamin D_3 to the drinking water, and the contract grower top-dressed the feed with 75 pounds of calcium phosphate. The mortality on day 17 was 36, but increased to 1500 on day 18. Feed was removed on day 18 and mortality declined rapidly. No lesions were reported at necropsy on day 18; 3-nitro-4-hydroxy phenylarsonic acid and/or salinomycin toxicity was suspected.

Histologic lesions in tissues collected at day 18, however, were severe nephrosis with loss of renal tubular parenchyma and large urate deposits, some with giant cell macrophages at the periphery. Ureters were distended with urates. Livers were congested, had necrosis of individual hepatocytes, heterophilic inflammation, mild coarse vacuolar change, and major bile ducts contained casts of bile.

The history and lesions were consistent with intoxication, with lesions in the kidneys causing the high mortality. Vitamin D_3 toxicity was suspected due to histologic lesions in the kidney (see reviews 1, 4). The history and lesions in this case were suggestive of one or more contributing factors, including excessive vitamin D_3 administration, concurrent feed additive toxicity from 3-nitro or salinomycin, possible inadequate water intake due to summer heat or

recumbency causing an exaggerated toxicity, or the calcium and phosphorus as a top dress on the feed being a contributing nephrotoxic factor.

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IT'S NOT THE PROGRAM, IT'S THE EXECUTION!

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Poultry technical service veterinarians have only a day, or perhaps two at a complex and little opportunity to conduct proper trials or diagnostic surveys. To obtain hard data, they rely heavily upon diagnostic work from local laboratories and the production numbers shared by the complex. They do not have the ability to follow a problem closely with careful data collection and proper diagnostic follow-up. So what good are they, anyway?

Technical service veterinarians have a unique tool: the power of observation. Poultry health problems are composed of complex interactions between environment, nutrition, husbandry and pathogens. The heavy travel requirement of a technical service veterinarian gives them the unique opportunity to juxtapose different sets of variables from multiple integrators. They can use their broad observations to recognize a *forest* when the local managers are trying to identify each tree. The following are my observations and opinions, after 20 years of traveling and sorting through poultry health problems.

Major poultry health program changes are best used to solve "simple" problems, for instance, the introduction of a new pathogen like infectious laryngotracheitis virus. In this case, altering the vaccination program to include an ILT vaccine may immediately reduce losses.

Unfortunately, major program changes are often used for the wrong reasons. Most poultry health issues start as small, nagging problems on widely scattered farms. They quietly smolder until a rise in fuel prices, a rise in corn prices, complex expansion or a change of seasons triggers more widespread outbreaks. Suddenly the pressure is on and the manager needs to "do something". Radical changes in the poultry health program usually compound and obscure the original problem, making it even more difficult to regain control of poultry health.

In many cases, the problem was never the poultry health program as it was designed. It was the *execution* of that program. "Execution" may be something as simple as the details of how a vaccine is applied under field conditions. It may also be a more complex interaction between the environment, management, nutrition and vaccination. This is why side-by-side integrators with intermingled farms can have very different disease problems while using the same vaccination program.

I specifically mentioned four common triggers for sudden widespread outbreaks. Usually the outbreaks take the form of respiratory disease or "leg problems", although enteric disease and poor performance may also result. Seasonal changes affect ventilation, humidity and temperature variation within a poultry house or hatchery. High fuel prices mean reduced ventilation, higher humidity and prolonged high-density, half-house brooding. High corn prices mean pressure on the nutritionist to reduce feed cost by substituting cheaper ingredients or buying lower quality corn. And complex expansion often means short down-time, high stocking density, and personnel, feed mills and infrastructure that are stretched to the limit.

Lighting programs, immunosuppressive diseases (such as the peak months of ALV-J) and reduced-cost ration formulation can also induce widespread breakdowns in flock health. These components may result in "stress" and "immunosuppression" – relatively undefined yet highly significant factors in poultry health.

Why can these common things trigger widespread disease outbreaks? Health programs depend upon a balance of multiple systems. If most systems are running well, one or more of the components can be substandard without a noticeable effect on performance. The integrated poultry industry plays the game of balance as a good business practice. If health is good and management is good, feed cost can be reduced by feeding fewer medications or by feeding lower protein or cheaper ingredients. If performance isn't affected, the integrator can further reduce cost by decreasing the time and headcount needed to apply vaccines. More production cost can be shaved by reducing the headcount needed to manage the farms. In the end, a highly profitable company may have shaved each component to the limits without affecting performance – until the occurrence of one of the major trigger events.

*Let's look at a single "trigger" example: fuel cost***.** An integrator grows a 5.5 lb bird placed at 0.78 ft^2. Their respiratory vaccination program is B_1-Mass-Conn vaccine via spray at the hatchery and field boost via spray using a back-pack sprayer at 17 days. The flock supervisor vaccinates the flock at 17 $\pm$ 2 days with the help of the grower, when the grower is available. Birds are half-house brooded to 14 days in the winter, and released between day 7 and day 10 in warmer weather.

The bird health was great for the past three years. The air sac condemnations would rise a bit during the erratic weather of early winter and spring, but once temperatures settled down, condemnations would predictably drop.

This year, condemnations began to rise in November and growers began to call for medication in December. In January, the complex was in the midst of a full-scale respiratory disease problem and ELISA data showed high and erratic infectious bronchitis titers. Samples have been sent to a laboratory for PCR, but no data is available yet. The top production manager has asked the veterinarian to add Arkansas, 072 or Georgia 98 strains of IB to the vaccination program.

What really happened? The fuel prices went up dramatically in September. The individual broiler growers did what they could to manage fuel costs: some held birds in the half-house to day 15, or even as late as day 17 during very cold weeks. The flock supervisors often didn't know that the birds had been held extra time unless they were still in half-house on vaccination day. Some flocks were moved at day 14, but the back of the houses weren't pre-warmed as well, so the birds didn't move out properly. Some flocks were field boosted in the half house, some in full house. Most houses had wet floors on the brood end from being held at higher density for a longer time, and some were caked throughout due to reduced ventilation. Litter quality was worst in houses that had cleaned out in late fall because litter prices had been driven up by fuel cost, and growers only covered the floors with an inch or two. Temperatures were a bit cool and humidity was high in many houses. Ammonia levels were up in most houses. Flock supervisors were stretched thin by sick bird calls, and had less time than usual to complete the field boost vaccination. Most farms were vaccinated at the rate of five minutes per house.

The original program was marginal. It worked because the birds were in full house prior to field boosting and ventilation was sufficient to manage litter quality and temperature except at the worst times of the year. The repercussions of the higher fuel costs – concentration of birds (and viruses) in the half-house; stress due to wet litter, erratic temperature and ammonia; reduced vaccination coverage and consistency, and reduced management supervision – all contributed to an increase in disease.

Once widespread disease outbreaks begin, the first response is always to make major changes in the vaccination program. This rarely improves the situation, and often compounds it. Adding Arkansas IB vaccine in the situation above would be like adding gasoline to a fire.

Correction of the problem usually involves an analysis of each component to find and amend the short-cuts. Since feed cost has the greatest impact on overall production cost, improvements in feed ingredients are the last resort. Hatchery management is often one of the first areas to come under scrutiny,

although this is seldom the true root of the problem. Corrective efforts should begin with improving the *execution* of the brooding management, ventilation management, feed management and vaccination technique. The biggest challenge is convincing field managers that what they considered "normal" management were really short-cuts. It's hard to believe that proper execution takes more time and manpower unless you've seen it done elsewhere, and you've seen the results.

The fuel cost example is just an amalgam of several real situations. The cases that I present will be true field examples of the interaction of management, nutrition, vaccination and environment. Some will be supported by some scientific data, others are anecdotes supported by observation only. These cases are interesting reminders that it isn't enough for a poultry practitioner to understand avian anatomy and infectious, neoplastic, or metabolic disease. The basics of poultry medicine are important, but that's the equivalent of identifying and understanding the trees. To be effective poultry practitioners, we also have to be able to see and understand the entire ecosystem of the forest.

PATHOGENICITY OF TURKEY ASTROVIRUSES TYPE 2 IN POULTS

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The pathogenicity of five different type 2 turkey astroviruses (TAstV-2) was studied in SPF turkeys. These viruses belong to three different genotypes based on sequence analysis of the capsid gene (1). Poults were inoculated at one day of age and examined for clinical signs and virus shedding during the 14-day experiment. All isolates caused enteritis and growth depression; however there were differences in disease severity among the isolates studied. Virus shed was monitored by quantitative real time RT-PCR on cloacal and oral swabs and intestinal contents, at 3, 7, and 14 days post-inoculation (dpi), with the highest titers at 7 dpi. Virus was also detected in blood demonstrating systemic spread. Common macroscopic lesions observed at necropsy were distended intestines, with translucent walls, filled with watery contents and undigested feed, dilated ceca with foamy contents, and pale pancreas. Microscopic lesions present in the intestines consisted of villous atrophy and lymphocytic infiltration in the intestinal submucosa. Presence of viral antigens was demonstrated by immuno-histochemistry in both villi and crypt enterocytes in the jejunum and, less frequently, the duodenum. Mild lesions were also observed in other organs including bursa, pancreas, spleen, liver, and kidneys, the extent of these lesions varying depending on the virus isolate genotype. In conclusion, genotypically different TAstV-2 viruses produce similar enteric diseases in turkeys; however the severity of the disease varies depending on the virus. The role of the capsid gene in TAstV-2 pathogenesis needs to be further investigated.

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MOLECULAR INVESTIGATIONS ON TURKEYPOX VIRUS

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SUMMARY

In Germany avian pox was diagnosed between 1999 to 2005 in seven turkey flocks. To characterize these turkeypox isolates molecular biological investigations were carried out with German isolates as well as with turkeypox isolates obtained from other countries. Results obtained by PCR in combination with restriction enzyme analysis and verified by sequence analysis revealed that turkeypox isolates from various countries, except one isolate from Chile, showed no differences in comparison to fowlpox virus (FWPV). The uniformity to FWPV was further supported, by the detection of different REV sequences in the investigated turkeypox isolates, since it is known that reticuloendotheliosis virus (REV) sequences are present in FWPV, whereas other avianpox virus species seem to be free.

Avian pox is a worldwide distributed viral disease in a broad range of wild, captive, and domestic birds, including chicken and turkey. The causative agents avian poxviruses are large DNA viruses, classified in the genus *Avipoxvirus* within the subfamily Chordopoxvirinae in the family Poxviridae. Fowlpox virus (FWPV) is the prototype of the genus *Avipoxvirus* that comprised currently nine further defined species, such as turkeypox virus (TKPV), pigeonpox virus (PGPV), or canarypox virus (CNPV), etc. The genome of avian poxviruses consists of a single molecule of linear double stranded DNA varying in size from 260 to > 300 kilobase pairs (kbp) and for some years the integration of reticuloendotheliosis virus (REV) proviral DNA into the genome of FWPV has been known (1, 3, 8, 9). REV, an avian retrovirus, is associated with immunosuppression and neoplasia in poultry and other avian species. Interestingly, whereas FWPV field isolates carry a nearly full intact REV provirus, most FWPV vaccine strains contain only a REV long terminal repeat (LTR) remnant (1, 6).

In poultry, poxvirus infections are often associated with significant economic losses by drop in egg production, reduced growth, and increasing of mortality. Due to differences in management, hygiene, and vaccination regime in different areas the incidence of this disease is variable. In Germany, infections with avipoxviruses have never been recorded over a period of 25 years in commercial poultry farms. However, since 1999 several outbreaks of avian pox were observed in different regions of Germany (4). In front of this background, the present study deals with epidemiological investigations on poxvirus infection especially in turkey flocks and in particular with molecular characterization of acquired German turkeypox isolates in comparison with isolates from other countries using different molecular biological tools.

MATERIALS AND METHODS

Diagnosis of avipoxvirus infection. Beginning in 1999 samples (skin, trachea) derived from chicken and turkey flocks suspected of poxvirus infection were investigated by polymerase chain reaction (PCR) for presence of avipox specific DNA. The PCR was carried out using a primer pair primarily designed for specific detection of FWPV (5); however, the primer pair was also useful to amplify DNA from various avipoxvirus species (7).

Molecular characterization. For molecular characterization seven isolates obtained from German turkey flocks as well as four further turkeypox isolates received from Brazil (two isolates), Croatia (one isolate), and Chile (one isolate) were included in the investigations. For species differentiation isolates were investigated by restriction enzyme analysis (REA) and sequence analysis of avipox PCR products as described previously (7). For further characterization isolates were screened for possible integration of REV sequences into their genomes. To address this issue a multiplex PCR for detection of avipox DNA as well as for four different regions of REV proviral DNA was performed (2, 6). In addition, a chimeric PCR was used to confirm, if REV proviral DNA was integrated in their genomes. For this PCR a forward FWPV primer was chosen from FWPV sequences flanking the REV provirus integration site and combined with the reverse primer specific for the REV LTR region (6).

RESULTS AND CONCLUSIONS

From 1999 to 2005 avian pox was identified altogether in more than 60 chicken and turkey flocks showing the re-emerging of this important disease in Germany. On the other hand, since 2002 application of an intensive vaccination resulted in a noticeable

reduction in the number of outbreaks. In turkey flocks the prevalence was low particularly in comparison with layer flocks. Avian pox was diagnosed over the whole period only in seven out of 23 investigated flocks. In detail, the infection was detected in five out 12 tested turkey breeder and in two out of 11 tested commercial meat turkey flocks.

In order to characterize the obtained turkeypox isolates, further molecular biological investigations in comparison to isolates derived from other countries were carried out. Results of species differentiation by restriction enzyme analysis of avipox PCR products using the two different enzymes *Eco*RV and *Mse*I yielded for turkeypox field isolates from Germany, Brazil, and Croatia no differences among each other as well as in comparison to FWPV. Only the isolate received from Chile was clearly different from the other tested turkeypox isolates. In comparison with further avipoxviruses such as PGPV or CNPV all examined turkeypox isolates were distinguishable. Furthermore, the apparent genetic similarity between turkeypox isolates from Germany, Brazil, Croatia, and FWPV was confirmed by sequence analysis, whereas the isolate from Chile was genetically distinct from the other tested turkeypox as well as from other avipoxviruses.

In addition, investigations by multiplex as well as chimeric PCR indicated the integration of a near full length REV provirus into the genomes of the turkeypox isolates from Germany, Brazil, and Croatia, comparable to FWPV field isolates. In contrast, investigations of the isolate from Chile as well as other avipoxviruses suggested that they seem to be free of REV sequences. In conclusion, the demonstrated uniformity between turkeypox isolates from various countries and FWPV suggest that turkeypox viruses are identical to FWPV and represents no distinct species. To classify the isolate from Chile further investigations are necessary.

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MYCOPLASMA SYNOVIAE PNEUMONIA OF TURKEY BREEDER HENS

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ABSTRACT

Turkey breeder hens showed an increase in mortality beginning at eight weeks of lay with no other clinical signs or changes in egg production. While no respiratory signs were observed in live turkeys, those that died consistently had gross lesions of pneumonia. Histopathology of lungs revealed serofibrinous bronchopneumonia, lymphofollicular reaction, and other features suggesting a bacterial etiology. However, except for incidental findings bacteria were not visualized in the sections examined, and none were isolated in meaningful numbers on routine bacteriologic media. At 12 weeks of lay the flock showed serologic evidence of infection with *Mycoplasma synoviae* (MS), and MS was identified by both mycoplasma culture and polymerase chain reaction (PCR) procedures in samples from choanal clefts and tracheas. Results of lung histopathology and PCR tests were consistent with a diagnosis of pneumonia caused by MS.

MYCOPLASMA SYNOVIAE AND COMMERCIAL LAYERS *E. COLI* PERITONITIS SYNDROME

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Mycoplasma synoviae (MS) is an important pathogen of domestic poultry, and is prevalent in commercial layers. During the last decade *Escherichia coli* (*E. coli*) peritonitis became a major cause of layer mortality. The association between the introduction of MS into young layer flocks and *E. coli* peritonitis was suspected. The possible role of MS in the laying hens *E. coli* peritonitis syndrome was studied. Four groups of mycoplasma free commercial layers at the onset of lay (at about 80% daily egg production) were challenged with a virulent MS strain or a virulent avian *E. coli* strain or both. The MS was applied by aerosol and the *E. coli* was applied intratrachealy three days post MS challenge. The four experimental groups contained 64 birds each, and were designated: negative control, *E. coli*, MS, and MS plus *E. coli*. A typical *E. coli* peritonitis mortality was reproduced in both of the *E. coli* challenged groups. *E. coli* peritonitis was the only cause for the post challenge mortality and included 1, 3, 0, and 5 birds in the negative control, *E. coli*, MS, and MS plus *E. coli* groups, respectively. The higher mortality in the MS plus *E. coli* group relatively to the negative control group was statistically significant, while the *E. coli* group did not have statistically significant higher mortality than the negative control group. The mortality differences between the *E. coli* and the MS plus *E. coli* groups were not statistically significant. Four weeks post challenge ten clinically normal birds from each of the four experimental groups were necropsied. All the birds in the two MS challenged groups demonstrated severe tracheal lesions. Body cavity lesions were detected in two and four birds in the MS and MS plus *E. coli* groups, respectively, with more prominent lesions in the MS plus *E. coli* group. The sole involvement of the challenge strains in the experiment was demonstrated by *E. coli* Pulsed-Field Gel Electrophoresis and MS *vlhA* Single Locus Sequence Typing. The results demonstrate a possible pathogenesis mechanism of respiratory origin to the layers' *E. coli* peritonitis syndrome, and suggest that a virulent MS strain can act as a complicating factor in this syndrome.

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

THE *MYCOPLASMA GALLISEPTICUM* 16S-23S rRNA INTERGENIC SPACER REGION SEQUENCE AS A NOVEL TOOL FOR EPIZOOTIOLOGICAL STUDIES

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Mycoplasma gallisepticum (MG) contains two sets of rRNA genes (5S, 16S and 23S) in its genome, but only one of the two is organized in an operon cluster and contains a unique 660 nucleotide intergenic spacer region (IGSR) between the 16S and the 23S rRNA genes. A polymerase chain reaction (PCR) was designed for the specific amplification of the complete MG IGSR segment. The MG IGSR PCR was tested on 18 avian mollicute species and was confirmed MG specific. The reaction also differentiated between MG and *Mycoplasma imitans* with a significant size difference in the IGSR of these two closely related avian mycoplasmas. The reaction sensitivity was demonstrated while comparing it to the well-established MG *mgc2* PCR. The MG IGSR sequence was found to be highly variable (discrimination (*D*) index of 0.950) among a variety of MG laboratory strains, vaccine strains, and field isolates. It was noted that none of the study's strains and field isolates shared the ts-11 vaccine strain MG IGSR sequence. The sequencing of the MG IGSR appears to be a valuable single-locus sequence typing (SLST) tool for MG isolate differentiation in diagnostic cases and epizootiological studies.

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

GENOMIC SIMILARITIES OF APEC SEROTYPE 078 STRAINS ISOLATED IN EPISODES OF COLISEPTICEMIA IN BREEDERS AND FINISHING MEAT TURKEYS IN ITALY

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INTRODUCTION

Colibacillosis is considered one of the leading causes of economic loss in the poultry industry worldwide (9). Serotypes O1, O2, and O78 are present in 15% to 61% of turkey colibacillosis cases. In Italy, *Escherichia coli* (*E. coli*) O78 is the most prevalent serotype isolated from turkeys according to D'Incau *et al*. (2). Although *E. coli* is likely widespread in the normal gastrointestinal flora of poultry, it is thought that only specific strains are endowed with virulence factors enabling them to cause disease (8). These strains, known as avian pathogenic *E. coli* (APEC), are able to cause diseases such as coliform omphalitis, yolk sac infection, colisepticemia of respiratory and enteric-origin, cellulitis, and swollen head syndrome (1).

Among the virulence factors involved in the pathogenesis of colisepticemia: 1) F1 type 1 fimbriae (*fimC*) adheres to the chicken respiratory epithelial cells of the pharynx and trachea; 2) temperature-sensitive haemagglutinin (*tsh*) plays a role in the colonization of air sacs; 3) the aerobactin iron-sequestering system (*iucD*) allows *E. coli* to grow in liquids with low concentration of free iron such as blood; and 4) *P fimbriae* (*papC*) are important in giving bacteria resistance to phagocytosis (4). Finally, two genes, *irp-2* and *fyuA,* are involved in iron uptake mechanism essential for bacteria entering the bloodstream (3). In a previous study, Giovanardi *et al.* (4) demonstrated the transmission APEC from chicken broiler breeders to their one-week old progeny. *E. coli*

with high genomic similarities was isolated from both breeders and their chicks that died of colisepticemia and omphalitis.

The purpose of this study was to determine the genomic similarity of APEC serotype O78 strains isolated from breeders and commercial meat turkey farms in Italy. This was conducted in Veneto, a region located in northeast Italy, in a fully-integrated poultry production company.

MATERIALS AND METHODS

Samples. In August and September 2006, seven cases of colisepticemia in breeders and 12 cases from meat turkeys were submitted to Laboratorio Tre Valli. All one-day-old breeder poults came from the same genetic company. Table 1 shows the farms involved and the ages of chickens used in this study.

Bacteriology and serotyping. Samples from all submissions were taken from the brain, air sacs, pericardial sac, lungs, and joints and were cultured into 3% sheep blood agar and incubated aerobically at 37°C for 18 to 24 hr. Suspect *E. coli* were subsequently inoculated on eosin – methylene blue agar (EMB, OXOID, Basingstoke, UK) and incubated for the same time and temperature as described previously. The identification of *E. coli* was based on the results of diagnostic tests, which included Gram stain, catalase, oxidase, and inoculum on triple iron sugar agar (OXOID). Biochemical profiles were analyzed for each isolate using the API system (Bio Mèrieux, France) designed for the identification of *Enterobacteriaceae*. Serological characterization was carried out using the slide agglutination test using O78:K80 antisera (Veterinary Laboratories Agency, UK).

Antibiotic sensitivity test. A sensitivity test (Kirby-Bauer) for seven microbial agents frequently used in local turkey flocks was performed on all the strains by the standard disk procedure as recommended by NCCLS (7). A four-hour broth culture was prepared for all the isolates and swabbed on the surface of Mueller-Hinton agar (OXOID). Ampicillin, amoxicillin, oxytetracycline, gentamycin, trimethoprim-sulfamethoxazole, ceftiofur (OXOID) and enrofloxacin (Bayer, Leverkusen, Germany) standard paper disks were laid on the medium. The plates were incubated for 24 hr at 37°C and inhibition zones were measured by SIRSCAN 2000 (i2a, Montpellier, France), an automated image analyzer.

Polymerase chain reaction (PCR). *E. coli* strains were inoculated into EC broth (Difco, Detroit, MI, USA), incubated aerobically at 37°C for 12 hr and then submitted for PCR. DNA was extracted with DNeasy Tissue kit (Qiagen, Germany) as described by the supplier. Amplification was done with Qiagen Multiplex PCR kit (Qiagen) in 25 µL reaction mix containing 5 µL bacterial DNA (20 ng/µL) and 200 µM primers for detection of each virulence associated genes: *iucD, tsh, papC, fimC* (5), *fyuA* and *irp-2* (3). Reaction conditions used were: 15 min denaturation at 94°C; 30 cycles of 30 sec each at 94°C; 90 sec at 57°C; 1 min at 72°C; and finally a cycle at 72°C for 10 min. The PCR products were separated by electrophoresis on 2% of agarose gel stained with SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA).

Random amplified polymorphic DNA (RAPD). Bacterial DNA was extracted with Prepman Ultra (Applied Biosystems, CA, USA) as described by the supplier and quantified by spectrophotometer. 20 ng of DNA were used as template in the RAPD kit containing room-temperature stable dried Ready-to-Go beads (Amersham Biosciences, Little Chalfont, UK). The kit was used as described by the supplier with primer 1290 (6). Reaction conditions used were: 5 min denaturation at 95°C; 45 cycles of 1 min each at 95°C; 1 min at 36°C; and 2 min at 72°C. Amplification products were resolved by electrophoresis on 2% agarose gel and detected by SYBR safe DNA gel stain. A 100 bp molecular marker (Invitrogen) was used. The image was captured using Gel Doc 2000 (BIO-RAD, Herchules, CA, USA) and saved as .TIFF files. The samples were analyzed in two independent reactions to detect reproducibility of the test and some reactions were run without primers or template. The fingerprinting was analyzed with Gel Compare II (version 2.0, Applied Maths, Belgium) and the measure of the similarity was based on densiometric curves using Pearson correlation (product moment correlation coefficient). A dendogram was generated by unweighted pair group method with arithmetic average (UPGMA).

RESULTS

Post-mortem. In all cases submitted, severe post-mortem lesions including pericarditis, airsacculitis, and fibrinous pneumonia were observed. These were highly suspicious of colisepticemia due to pathogenic *E. coli*.Culture, biochemical, and serological characterization. Nineteen different types of *E. coli* were isolated from the farms involved as summarized in Table 1. All were oxidase negative, non hemolytic, catalase and lactose positive, with characteristic greenish-black metallic sheen on EMB agar. The API commercial differentiation system identified all the isolates as *E. coli*. Serotype O78 was mainly isolated from brain (84%), lungs (47%), pericardial sac (32%), and joints (16%). The antibiotic sensitivity test on the 19 *E. coli* O78 isolates showed a high level of resistance to three antimicrobial agents, namely, ampicillin, amoxicillin, and oxytetracycline; and

sensitivity to trimethoprim-sulfamethoxazole, gentamycin, ceftiofur, and enrofloxacin.

PCR. PCR for the presence of virulence factors was performed for all 19 *E. coli* O78 isolates. Virulence associated genes *iucD, tsh, fimC*, *irp-2* and *fyuA* were present in all strains isolated and all were negative for *papC* gene. In our analysis of the PCR data, we assumed that all *E. coli* O78 belonged to the same pathotype.

RAPD. RAPD technique was used to analyze the cloned relationships of the *E. coli* isolated. The information from the phylogenetic analysis was used to determine if there were any genetic similarities between the *E. coli* O78 of the same pathotype and pattern of antibiotic resistance. The RAPD analysis using primer 1290 revealed eight distinct patterns. *E. coli* correlated with similarities in their RAPD DNA patterns were clustered (Fig. 1). The most interesting clusters were the following: 1) cluster I which consisted of *E. coli* O78 isolates A1 and A2 from layer breeders, and isolates E and F from meat turkeys; 2) cluster II which consisted of *E. coli* O78 B1 and B2 from layer pullets, C from layer breeders, and I, L, M from meat turkeys; and 3) cluster III with isolates D1 and D2 from pullet breeders and two isolates, N and O, from meat turkeys (See Table 1). To test the reproducibility of the RAPD technique, the samples were analyzed in two independent reactions. Results obtained showed no loss or shift in the position of banding patterns. When the reactions were ran without primers or templates amplification was not obtained (data not shown). Five of the *E. coli* O78 strains namely G, H, P, Q, and R isolated from commercial meat turkeys, did not belong to any of the three clusters, which may indicate that they are genetically different from the isolates in clusters I, II, and III (Figure 1).

DISCUSSION

In our study, the isolation of B1 and B2 from pullet breeders (7 weeks) suggests an early *E. coli* O78 infection of birds. Since they are highly similar to strain C from layer breeders, based on their cluster II correlation, we believe that strains B1, B2, and C may be derived from day-old poults coming from the same genetic company. In addition, we hypothesize those healthy layer breeders showing no signs of clinical colibacillosis harbored the pathogenic strains in their intestines since they were pullets. At oviposition, *E. coli* O78 may have infected eggs through fecal contamination of eggshells as demonstrated in our previous study in chicks (4). Our data showed that finishing turkeys of flocks "e" and "f" received day-old birds from breeder "a". Referring to the dendogram, the inclusion of A1, A2, E, and F *E. coli* strains in cluster I, is highly indicative of APEC transmission from breeders to their progeny (the genomic similarity of 95% in Pearson correlation is considered very high). Cluster III also shows genomic similarities between E. coli O78 strains from pullets (D1 and D2) and meat turkeys (N and O).

As observed in this study and those done by the company in the past, we observe a trend that cases of colisepticemia due to pathogenic *E. coli* O78 are rare in adult breeders. Although rare, it is possible that the bacterium may be more widespread in the layer breeder population than we expected. The data presented is preliminary and further work is needed to better elucidate the modality of transmission of APEC from turkey breeders to their progeny.

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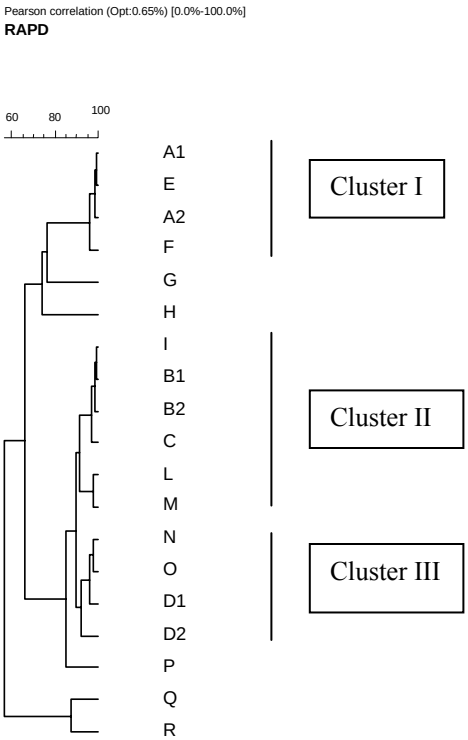
Table 1. A summary of the farms and turkeys sampled and the *E. coli* strains isolated.

	Farms	Age of turkeys	*E. coli* strains	Clusters from RAPD isolated correlation
Breeders	a	31 wk	A1 and A2	I
Pullets	b	7 wk	B1 and B2	II
Breeders	c	33 wk	C	II
Pullets	d	13 wk	D1 and D2	III
Meat turkeys	e	22 d	E	I
	f	20 d	F	I
	g	33 d	G	No Correlation
	h	25 d	H	No Correlation
	i	100 d	I	II
	l	62 d	L	II
	m	41 d	M	II
	n	70 d	N	III
	o	59 d	O	III
	p	50 d	P	No Correlation
	q	80 d	Q	No Correlation
	r	56 d	R	No Correlation

Figure 1. UPGMA dendogram constructed from RAPD using Pearson coefficient. The data indicate strict genetic similarity among the *E. coli O78* strains grouped together into clusters.



A RATIONAL ATTENUATED MUTANT OF AN AVIAN PATHOGENIC *ESCHERICHIA COLI* SEROVAR O78: A POSSIBLE CANDIDATE LIVE VACCINE STRAIN FOR THE PREVENTION OF AVIAN COLIBACILLOSIS

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INTRODUCTION

Control of colibacillosis is important to the poultry industry because the economic impact of this disease is overwhelming (1). *Escherichia coli* serovars O1, O2 and O78 are frequently isolated from birds affected with colibacillosis (4). Pathogenic *E. coli* causes a variety of disease manifestations including septicemia, peritonitis, granuloma, and cellulitis in poultry. Traditionally, antibacterial agents have been widely used for the treatment and control of *E. coli* infection in poultry flocks. However, residual antibiotics in poultry meats and the emergence of drug-resistant bacteria have led to increasing interest in alternative methods of protecting poultry flocks against colibacillosis. Killed oil-emulsion vaccines for breeder hens and commercial broiler chickens have been licensed in Japan, but their use is limited because of the difficulty of application. To develop a live vaccine that is more efficient, safe and easy to apply, we constructed an attenuated mutant of avian pathogenic *E. coli* O78 by an allelic exchange procedure. In this presentation, we characterized the mutant having a deletion in the cyclic AMP receptor protein gene (crp) in immunological and safety aspects and we evaluated the mutant for potential use as a vaccine strain.

MATERIALS AND METHODS

The parent bacterial strain used was the avian pathogenic *E. coli* strain J29 of serovar O78, which was originally isolated from a chicken with colisepticemia. Strain J29 showed comparatively low virulence for chickens and is sensitive to many kinds of antibiotics. Mutant AESN1331 was constructed from strain J29 using an allelic exchange system (2) with a host *E. coli* strain SM10 lambda pir and a suicide plasmid pCVD442 containing a small crp fragment with a 351 bp deletion at a central part of the gene. The antigenicity, size and color of colonies grown on an agar plate, sugar fermentation, adsorption of Congo-red, nucleotide sequence of the crp gene, LD50 values in five-week-old chickens, duration of the detection of the live bacteria in chicken organs after challenge by spraying, were examined using the mutant AESN1331 and parent J29 strains.

In the first experiment, 40 of newly hatched white leghorn SPF Line-M chickens were randomly allocated to three groups of 10 each. Each group was inoculated twice at ages of 4 and 32 days by spraying with fine particles (group 1) or coarse particles (group 2) or by eye drops (group 3). The remaining 10 birds were used as untreated controls. To evaluate safety, all groups of chickens were monitored daily to detect adverse reactions subsequent to the administration, and were serially weighed at administration and challenge to confirm normal weight gain. For the evaluation of efficacy, all experimental and control chickens were intravenously challenged with virulent *E. coli* serovar O78 strain at the 46 days of age. After the challenge, chickens were monitored daily for signs of illness and for deaths. One week after the challenge, surviving chickens were euthanatized and macroscopic lesions were recorded. For serological examination, blood samples were taken from all chickens just before the administration and at the time of challenge to determine the serum antibody level using an O78 LPS ELISA.

In the second experiment, 103 CFU of the mutant strain AESN1331 were inoculated into the amniotic fluid of eggs on the 19th day of embryo development. To evaluate the efficacy of *in ovo* application of the mutant, the chickens hatched from eggs with or without administration, were intravenously challenged at the age of 32 days with virulent *E. coli* O78. The safety and efficacy of the administration was examined as described in the first experiment.

RESULTS

The crp mutant of *E. coli* AESN1331 was agglutinated with anti-O78 rabbit serum, indicating the LPS structure of the mutant was not affected by the introduction of the mutation. Within 16 to 24 hr at 37°C, the mutant strain grew as a small colony on the trypticase soy agar plate and a colorless colony on the MacConkey agar plate. The mutant strain could ferment glucose, but could not ferment lactose or

xylose. The parent strain J29 had the ability to adsorb Congo-red, whereas the mutant AESN1331 could not.

The nucleotide sequence analysis of the crp gene demonstrated that the parent strain J29 has a complete crp gene comprised of 633 bp, but the mutant AESN1331 possessed a small crp fragment of 282 bp, indicating that the central 351 bp of the gene had been deleted. LD50 value of AESN1331 was approximately 10 times higher than that of the parent J29 (6.2 x 108 CFU vs 8.0 x 107 CFU).

After inoculation via spraying, the mutant AESN1331 was detected in the chicken organs for only three days subsequent to the challenge, while the parent strain survived in the chicken body until day 35 after the challenge.

In the first experiment, there were no adverse clinical signs or lack of weight gain in any birds after administration of the mutant AESN1331 twice. After the challenge exposure, 8/10 chickens in the control group died, while only 1/10, 0/10 and 0/10 chickens died in the fine spray group, coarse spray group and eye drop group, respectively. The antibody levels measured after the last administration of the mutant strain (day 46) demonstrated that antibody response was detected in all treatment groups but not in the control group. Lesion scores for pericarditis and perihepatitis were significantly lower in the treatment groups compared to those in the control group.

In the second experiment, all control chickens died after the challenge, in contrast all chickens treated with the mutant strain AESN1331 *in ovo* survived. Positive antibody response was found only in the treated group but not in the control group. Lesion scores for pericarditis and perihepatitis were significantly lower in the treated group than in the control group.

DISCUSSION

We constructed an attenuated mutant AESN1331 of the avian pathogenic *E. coli* serovar O78 using an allelic exchange procedure. The mutant strain showed a deletion in the crp gene, which is involved in the global regulatory network of gene expression in *E. coli*. It is well known that the crp gene plays an important role in utilization of a variety of carbon-containing substances, and the crp mutant could not ferment sugars other than glucose. Lately, the crp gene is also shown to be involved in the expression of virulence factors of *E. coli* including beta-hemolysin, which we described previously (3).

The present data indicate that the mutant strain showed markedly reduced virulence and decreased colonization ability in the chicken body. However, administration of the mutant strain via various routes evoked an effective immune response that could protect against the virulent wild type *E. coli* O78 strain.

These findings suggest that the mutant constructed here appears to be a suitable candidate for a live vaccine strain to protect chickens from colibacillosis by avian *E. coli* O78.

THE EFFICACY OF A MODIFIED LIVE *E. COLI* VACCINE USING A BROILER SKIN CHALLENGE

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INTRODUCTION

Escherichia coli induced cellulitis, also called infectious process (IP), is one of the leading causes of broiler condemnations at processing. The generally accepted mechanism for the development of cellulitis is via skin scratch inoculation. Thus, preventive measures focus on management techniques that diminish the incidence of scratches. Proper litter management can also influence the challenge load of resident bacteria. In a preliminary study (WPDC poster, 2007) we demonstrated that a compromise to the birds' natural immune defenses can make them more susceptible to cellulitis caused by subcutaneous (SQ) inoculation. We also saw that a very high challenge exposure can overcome the birds' defenses, even if they are fully intact. The goal of this study was to see if active immunization using a new modified live (aro-A) *E. coli* vaccine could protect broilers from an SQ inoculation with two different O78 challenge serotypes.

MATERIALS AND METHODS

SPF broiler chicks were divided at hatch into those that received a full dose of Poulvac *E. coli* by coarse spray and non-vaccinated controls. All chicks were housed in Horsfall isolator units (20 per) at Auburn University. There was a chamber of isolators for each two-week challenge treatment: 1) O78 Isolate #1 (7.2×10^7/bird, 2) O78 Isolate #2 (5.1×10^8/bird), and 3) No *E. coli* challenge. Each chamber houses three isolators. Isolators A and B contained birds vaccinated with Poulvac *E. coli* while isolator C contained non-vaccinated controls. Challenge was by SQ injection of 0.1 mL over the right breast muscle. At three weeks of age, all birds were examined for *E. coli* lesions, including cellulitis.

Cellulitis lesions were scored according to criteria previously established by Macklin and Norton: Grade 0 → no cellulitis lesion; Grade 1 → mild-focal (<2cm in diameter); Grade 2 → moderate-focal (cellulitis covers up to a quarter of the breast muscle); Grade 3 → moderate-diffuse (cellulitis covers half the breast muscle; there is severe muscle infiltration); Grade 4 → severe-diffuse (entire breast muscle is covered with cellulitis; severe muscle infiltration).

RESULTS

Both *E. coli* challenge isolates caused a significant incidence of both cellulitis lesions and internal lesions (colibacillosis), indicating that the inocula went systemic (hematogenous) in many of the birds challenged subcutaneously. Challenge Isolate #2 caused significantly more Grade-3 cellulitis lesions, which in turn resulted in a higher mean lesion score. Spray vaccination at hatch did not significantly reduce the overall incidence of cellulitis lesions (data not shown). However, vaccination clearly did reduce the incidence of birds with severe lesions (see Table 1). Vaccination also significantly reduced the incidence of colibacillosis after challenge with Isolate #2 and resulted in a numerical reduction against Isolate #1.

The body weight differences were not significant. However, there was a trend toward lower body weights in the challenged birds, especially the groups that were not vaccinated (2C and 3C). Mortality in the challenge control groups was twice as high as in the vaccinated/challenged groups, but this was not significant and, essentially, only a one-bird difference.

DISCUSSION

Isolate #2's ability to cause a higher incidence of Grade-3 cellulitis lesions might indicate it has greater potential to cause cellulitis. In fact, this particular isolate was recovered from a broiler flock suffering from high IP condemnations. However, Isolate #2 was also given at almost a full log (base 10) higher titer than Isolate #1. It is interesting to note that giving Isolate #2 subcutaneously at two weeks of age resulted in internal *E. coli* lesions (i.e., airsacculitis, pericarditis, perihepatitis) while in a previous study (WPDC poster, 2007) there were no internal lesions after a 25 day challenge with the same isolate at a similar titer level. Perhaps the difference in hematogenous spread was due to differences in immune competence between a 14-day-old broiler and a 25-day-old broiler.

The modified live (aro-A) *E. coli* vaccine significantly reduced the severity of disease, both under the skin and on the serosal surfaces of the heart, liver, and airsacs. Body weights of the vaccinated birds were not as suppressed as the challenge controls either. The incidence of cellulitis exceeded 85% in all challenged groups, including vaccinates, so it is very possible that the high challenge dose (over 8 logs-base 10) was too much for the two-week old birds to completely resolve. Despite such a high challenge titer, however, the vaccine did significantly limit the extent of the skin infections as well as the bacteria's spread to the body cavity.

To better mimic current field situations, future studies could: 1) target a lower incidence of cellulitis in the challenge controls by either lowering the challenge titer or challenging older chickens; and 2) give the immune system (and the vaccine) a more fair shake by monitoring lesions longer post challenge and thus allowing more time for lesions to resolve.

Table 1. Three-week findings after hatch-day *E. coli* vaccination and two-week *E. coli* challenge.

Room ID#	Group Description	Body weight (g)	BW supp.	Incidence of internal *E. coli* lesions	Mortality	Cellulitis lesions	
						Mean score	Grade-3 incidence
1	Negative Controls	724	--	0% a*	0%	0.00 a	0% a
2AB	*E. coli* vaccination + *E. coli* isolate #1 challenge	696	3.9%	39.5% b	2.6%	1.58 b	13.2% ab
2C	*E. coli* isolate #1 challenge	654	9.7%	50.0% bc	5.0%	2.11 c	40.0% c
3AB	*E. coli* vaccination + *E. coli* isolate #2 challenge	704	2.8%	39.5% b	2.6%	2.11 c	28.9% bc
3C	*E. coli* isolate #2 challenge	670	7.5%	67.0% c	5.6%	2.72 d	77.8% d

* Groups within a column not sharing a same letter are significantly different ($p<0.05$).

BACTERIAL PRODUCTS TO CONTROL POULTRY DISEASES

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The poultry industry in the US experiences excellent performance through improvements in genetics, nutrition, and advanced management techniques. The industry addresses most diseases problems through a variety of interventions including better sanitation, balanced nutrition, vaccinations, and therapeutic and sub-therapeutic levels of antibiotics through drinking water or feed. Today's poultry industry needs new technology and science based interventions to continue to aid and maintain in the control and reduction of disease challenges.

Animals have become more vulnerable to potentially harmful microorganisms such as *Escherichia coli* and *Clostridium* spp. In an attempt to control some of these challenges, the use of in-feed antibiotic based promotants at both therapeutic and sub-therapeutic levels has become widespread. However, partly as a result of various public health scares associated with the antibiotic resistant microorganisms, there is increasing consumer pressure to reduce the use of antibiotics in feed. This has logically led to an increased interest in other methods of enhancing poultry performance and helping the bird to withstand disease. Hence, biotechnology companies are increasing the investment of time and money to look at alternatives to maintain growth and performance in farm livestock. Agtech Products is a company that has invested significant resources into the biotech area and is focused on using scientifically selected microorganism for application into the poultry industry. One of Agtech's interests is using the body's natural defense mechanisms referred to as "colonization resistance" by Van der Waaij *et al*. (1). This is in reference to the existence of a population of non-pathogenic bacteria which are normally and naturally present within the gastrointestinal tract (GIT) of all domestic animals and birds. There is a delicate balance of beneficial and pathogenic bacteria in the GIT with many symbiotic and competitive interactions occurring between them (2). Several mechanisms by which the normal microbial population provides host protection have been proposed. These include: the production of substances such as volatile fatty acids, which inhibit multiplication of non-indigenous organisms, competition with non-indigenous organisms for nutrients present in limited supply, and competition

with non-indigenous organisms for available tissue attachment sites (3). Stimulation of the host immune system and production of antimicrobial factors are also proposed mechanisms. There may be multiple means to impact this gut microbial environment. One method is by the continuous addition of a bacterial culture to the feed. This is done as a way to prevent colonization by potentially pathogenic microorganisms while also enhancing the host's immune system.

Clostridium spp. is typically one of the most ubiquitous microorganisms in poultry production systems. As anaerobic bacteria, *Clostridium perfringens* and *Clostridium septicum* continue to become more prevalent in commercial poultry production. To control clostridial diseases, it is increasingly evident that one needs to better understand the gut microorganisms involved in the GIT that maintain the balance between beneficial flora and pathogens. Clostridial spores are typically found in high numbers in both the litter and the GIT of poultry. These organisms are capable of producing an array of extra-cellular enzymes and toxins that degrade host tissues. Many different antimicrobials are currently used in poultry production to promote bird growth or to treat diseases caused by microorganisms. Three of the more common drugs fed at sub-therapeutic doses for growth promotants are bacitracin, flavomycin, and virginiamycin. Other drugs, penicillin and either chlortetracycline or oxytetracycline are often administered to control clostridial disease breaks. Penicillin has proven to be the most effective antibiotic tested at inhibiting the growth of *Clostridium* spp. *in vitro*. This was not a surprise as it continues to be the on-farm drug of choice for the control of cellulitis outbreaks today.

Over the last several years Agtech Products has worked extensively with many producers to better understand the microorganisms involved in clostridial diseases of poultry (16). This work has revealed an incredible amount of genetic diversity within the species of both *Clostridium perfringens* and *Clostridium septicum*. Both organisms are often present in a bird experiencing cellulitis or gangrenous dermatitis. Interestingly, this work also suggests that multiple strains of each species are often involved. Another important observation is that clostridial bacteremia is almost always associated with these diseases. It is not unusual to observe bacteremia in seemingly healthy broilers or turkeys. Although *C. perfringens* and *C. septicum* have been the most commonly observed toxigenic clostridia, other species such as *C. cadaveris*, *C. sordellii*, and *C. tertium* have been identified as well. A better understanding of these organisms and the diseases they cause is a prerequisite for the development of new intervention strategies to control these diseases.

Avian pathogenic *E. coli* (APEC) comprise a specific subset of pathogenic *E. coli* that cause extraintestinal diseases of poultry (5). APEC are naturally present in the intestinal microflora of healthy birds and infections are enhanced or initiated by secondary environmental stress and host predisposing factors. Colibacillosis refers to any localized or systemic infection caused entirely or partly by avian pathogenic *Escherichia coli* (APEC) and is the most frequently reported disease in surveys of poultry diseases or condemnations at processing (8). Colibacillosis is a common systemic infection caused by APEC, and occurs most commonly as acute septicemia or subacute airsacculitis and polyserositis in chickens, turkeys, and other avian species (7).

APEC consist mainly of enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) serovars (6). Previous publications have identified numerous virulence factors associated with APEC. The *iss* (increased serum survival) gene has been found to contribute to complement resistance of a human *E. coli* isolate and its presence is strongly correlated with *E. coli* isolated from birds with colibacillosis (9). The *iuc*C (aerobactin iron sequestering system) gene allows bacteria to grow at low levels of iron (10). The *tsh* (temperature sensitive hemagglutinin) gene is an autotransporter that may contribute to the development of lesions within the air sacs of birds (11). The *cva*C (colicin ColV operon) gene codes for colicin V which cause membrane leakage in target cells (12). Using a multiplex PCR procedure that incorporates primers for these four genes has been useful for the identification of avian pathogenic *E. coli* in poultry (7).

Over the past several years Agtech Products has researched the prevalence, distribution, and diversity of APEC within large integrated turkey and broiler companies (13, 14, 15) This research has discovered a large amount of diversity within the APEC communities both between sites as well as within sites at many of these companies. The level of APEC can range from 10^3 to 10^6 CFU/g or higher in younger birds. Research also indicates that similar APEC isolates can have different combinations and number of virulence factors associated with them. In general, APEC has been found to be more prevalent and more virulent among younger birds. These discoveries will allow the industry to find new interventions to aid in the control of disease challenges.

Agtech Products, Inc. has performed extensive research on APEC and clostridial diseases and now is developing *Bacillus* and *Lactobacillus* interventions that are effective in controlling these bacterial challenges.

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NECROTIC ENTERITIS: OCCURRENCE WITHOUT COCCIDIA

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SUMMARY

Baking soda ($NaHCO_3$) incorporated at a rate of 37.8g/454g (8.3%) in a commercial non-medicated chicken ration and provided 48 hr prior to being given an anaerobic cooked meat broth culture of *Clostridium perfringens* (10^7 logs cfu/mL). Birds developed acute enterotoxemia within 36 hr post inoculation; birds had classical signs and lesions with a 10% mortality due to necrotic enteritis. Histopathological findings were diffused, multifocal or focal necrosis.

INTRODUCTION

Necrotic enteritis is an acute enterotoxemic condition of young chickens and turkeys, often associated with high sudden mortality for a short duration. This disease was first reported in 1961 from a flock of cockerels from England (6). Since then, the disease has become relatively prevalent in commercial broiler production; however, in recent years because of the complete removal or reduced usage of in-feed antibiotics from poultry feeds there has been a bloom in the prevalence of necrotic enteritis in commercial poultry operations.

The causative agent of necrotic enteritis is a Gram-positive obligate anaerobic bacterium, *Clostridium perfringens*. Mortality may range from 2-50% which usually take place over a short period of time. Manifestation of enterotoxemia usually follows alterations of the microenvironment of the intestines due to physical, chemical, or physiological modifications. High dietary levels of animal protein such as fish meal or poor quality animal by-products, grain such as wheat, barley, oats, or rye encourage the proliferation of *Clostridium perfringens* in the small intestines. Coccidia have been implicated as one of the major contributors that initiate the damage encouraging the proliferation of CP in the intestines (2).

Signs of the NE are depression, ruffled feathers, and diarrhea. Gross lesions are usually found in the small intestine (jejunum) but have been seen in the large intestine and cecum. The classic lesion of NE is the mucosa covered with a tan to yellowish pseudo membrane "Turkish towel" and having a characteristic foul odor.

Several papers have indicated coccidia species, such as *Eimeria acervulina, E. maxima*, and *E. brunetti* are associated with NE (1, 2, 3, 7). In several NE research models, *E. maxima* is used as the agent to cause the initial insult to the intestines. In a recent report, Mathis and Hofacre (5) stated that chickens are more susceptible to the development of NE if infected with *Eimeria* and then exposed to *C. perfringens*, but the risk is greater with *E. maxima* than with *E. acervulina*.

Sodium bicarbonate has shown to have good benefits to be used in poultry diets: reduced water intakes, thus less wet houses; augment the efficacy of ionophores; and better meat yield (4).

METHODOLOGY

During an evaluation of a research model in isolation cages, to create necrotic enteritis (NE) in young broiler chickens, birds were given or not given coccidia at 22 days of age. Birds that received coccidia were given 50,000 sporulated oocysts of *E. maxima* each via gavage at 22 days of age followed by oral inoculation of a 4 mL anaerobic cooked meat broth culture of *C. perfringens (*10^7 log cfu/mL) 5 days post coccidia inoculation. The birds that had not received the coccidia, at 25 days of age, were provided with a special blend of feed (baking soda incorporated). Baking soda ($NaHCO_3$) was added to the basal diet at a rate of 83g/Kg (8.3%). These birds were also given a 4 mL anaerobic cooked meat broth culture of *C. perfringens (*10^7 log cfu/mL) *per os* at 27 days of age.

RESULTS AND DISCUSSION

Within 36 hr post inoculation with *Clostridium perfringens*, birds had developed acute enterotoxemia and with classical signs and lesions. This model produced acute enterotoxemia in young broiler chickens. Mortality rate among those birds that were given the baking soda concoction averaged 10%. Histopathological findings were diffused, multifocal or focal necrotic lesions; these were similar to those of birds having NE but with concomitant coccidial infections. NE appeared to begin at the tips of the villi and spread to the upper half of the mucosal surface. A common sign seen with the use of the baking soda was loose droppings (physiological diarrhea).

Birds fed a diet fortified with 8.3% $NaHCO_3$ (baking soda) and given *Clostridium perfringens* developed clinical signs and lesions of enterotoxemia.

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SENSITIVITY AND FLOCK PERFORMANCE COMPARISON OF NICARBAZIN-CONTAINING ANTICOCCIDIALS

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INTRODUCTION

There are only two anticoccidial products that contain nicarbazin and are approved for use as feed additives in broiler chickens in the United States (1), one contains approximately 113 g of nicarbazin per 454 g of premix (Nicarb) and the other one contains an equal amount of nicarbazin+narasin (36 g of each) per 454 g of premix (Maxiban). In a previous report (2), the sensitivity of 15 field isolates of mixed coccidial species to Nicarb and Maxiban showed that the field isolates were more sensitive to Nicarb than Maxiban. These findings were based on anticoccidial sensitivity tests (AST). The procedure for conducting AST has been described before (3). Briefly, in the AST, coccidia-free broiler chickens raised in wire floor cages and fed diets containing the corresponding anticoccidial are orally challenged with a measured dose of sporulated coccidial oocysts at 14 days of age. Six days post challenge the birds and feed are weighed in order to calculate average weight gain, feed consumption and feed conversion and the birds are euthanized and its intestines removed and individually scored for gross coccidial lesions by the method of Johnson and Reid (4). The results are compared to the results obtained in replicates of broiler chickens that were challenged but were not fed any anticoccidial drug. The main parameters analyzed include: weight gain, feed conversion, mortality and intestinal lesion scores. Since the previous report (2) which detailed anticoccidial sensitivity results from 15 field isolates of mixed coccidial species from broiler-producing areas across the United States, an additional 11 field isolates have been evaluated by the same procedure and were added to the original 15 and will be presented in the results section of this manuscript.

To determine whether comparative flock performance would be consistent with the results of the previously conducted Nicarb vs. Maxiban AST studies, a follow-up 38 day-long floor pen experiment was conducted under conditions of natural challenge (built-up litter). The findings of this experiment will also be presented in the results section of this manuscript.

MATERIALS AND METHODS

Sensitivity Tests

Collection of field samples. In addition to the fifteen previous isolates, litter samples were collected from 11 broiler complexes in 2005 and 2006 representing a cross section of commercial broiler-producing companies in the United States. Samples were obtained from houses from the upper one-third of the litter representing the brood end of each house from flocks between 21-28 days of age. A one pound composite sample from each house was placed in a plastic bag, put on ice and submitted overnight to Southern Poultry Research, Inc., of Athens, Georgia.

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

Description of *in vivo* study. The study consisted of a series of 11 battery tests and was conducted from January 2005 to July 2006. Male Cobb x Cobb chicks were raised in coccidia-free Petersime battery units to 12 days of age. Assignment of treatments to cages was by use of a random numbers table. Cages were blocked by location in the battery with block size equal to treatments by isolate (three cages per block). There were three cages per treatment and eight chicks per cage for a total of 24 chicks per treatment. Treatments consisted of chicks fed diets containing either Nicarb (113 g/909 kg) or Maxiban (72 g/909 kg), or a non-medicated control. Batteries were in an environmental structure with even illumination and a stocking density of 0.06 m^2/ bird.

Procedure for *in vivo* study. On day 12, day of test zero (DOT 0), chicks were weighed by cage. On

day 14 (DOT 2) all chicks were infected orally by pipette with one mL of isolate suspension containing 100,000 sporulated oocysts. On day 20 (DOT 8) the trial was ended and birds were weighed by cage and total feed consumption by cage was determined. Birds were euthanized and lesion scored by cage according to the method of Jonnson and Reid (4). Lesion scores were determined for upper, middle, and cecal regions of the intestine and scores reported as average of all regions.

Floor Pen Experiment

A total of 12 floor pens, each containing 30 male and 30 female Cobb x Cobb broiler chickens were assigned to each of two treatments. Both treatments were fed identical starter, grower, and finisher feeds; the only difference was that in treatment #1 Nicarb was included at 125 ppm in the starter feed whereas in treatment # 2Maxiban was included at 79.2 ppm. Other feed additives included in both starter treatments included 50 ppm of roxarsone and 55 ppm of bacitracin. The grower feed for both treatments was supplemented with 110 ppm of monensin, 50 ppm of roxarsone and 55 ppm of bacitracin. The finisher feed for both treatments was supplemented with 11 ppm of virginiamycin. The analyzed concentrations of all drugs were within the permitted analytical variance.

The starter feed was fed from 0-18 days, at which time the feed and chickens in all pens were weighed to calculate average body weights and feed conversions. From 18 to 31 days the birds were fed the grower feeds according to treatment; finisher feeds were provided from 31 to 38 days. At 38 days, all the remaining feed and birds in each pen were weighed in order to calculate final body weights and feed conversions. Feed and water were provided *ad libitum* for the duration of the test. The experiment was terminated at day 38.

STATISTICAL ANALYSIS

Analysis of variance and means separation for statistical significance were conducted by established procedures (5, 6).

RESULTS

Sensitivity Tests

A summary of the average results of the sensitivity tests for the combined 26 field isolates is presented in Graph 1. There were no statistically significant differences ($P>0.05$) for mortality and therefore the data are not shown.

There were highly significantly ($P<0.001$) differences between treatments in terms of weight gain, the challenged birds that were fed the unmedicated diet gained significantly less weight than those birds fed either the Nicarb or Maxiban supplemented diets. The broilers fed the diets supplemented with Nicarb had significantly greater weight gains than the broilers fed Maxiban.

There were also highly significantly ($P<0.001$) differences between treatments regarding feed conversion, the challenged birds that were fed the unmedicated diet had significantly higher feed conversion than those birds fed either the Nicarb or Maxiban supplemented diets. The broilers fed the diets supplemented with Nicarb had significantly better feed conversion than the broilers fed Maxiban.

Finally, there were also highly significantly ($P<0.001$) differences between treatments regarding intestinal lesion scores, the challenged birds that were fed the unmedicated diet had significantly higher intestinal lesion scores than those birds fed either the Nicarb or Maxiban supplemented diets. The broilers fed the diets supplemented with Nicarb had significantly lower intestinal lesion scores than the broilers fed Maxiban.

Floor Pen Experiment

A summary of the average results of the floor pen experiment is presented in Table 1. Since the mortality was within normal range and there were no statistically significant differences ($P>0.05$) between treatments, the data are not shown.

There were statistically significant ($P<0.05$) differences between the treatments in terms of weight gain. At the end of the starter phase, the broilers fed the diets supplemented with Nicarb were significantly ($P<0.05$) heavier than those fed the diets supplemented with Maxiban (550 *vs.* 506 g, respectively). At the end of the grow-out, the broilers fed the diets supplemented with Nicarb were also significantly ($P<0.05$) heavier than the broilers fed Maxiban (2.045 *vs*. 1.997 kg, respectively).

There were also statistically significant ($P<0.05$) differences between the treatments in regards to feed conversion. At the end of the starter phase, the broilers fed the diets supplemented with Nicarb had significantly ($P<0.05$) lower feed conversion (1.477 *vs*. 1.564, respectively). At the end of the grow-out, the broilers fed the diets supplemented with Nicarb had also significantly ($P<0.05$) lower feed conversion than the broilers fed Maxiban (1.760 *vs*. 1.797, respectively).

CONCLUSIONS

Results of anticoccidial sensitivity tests (AST) conducted with 26 mixed isolates of coccidia from 26 commercial broiler production complexes from various regions of the United States comparing Nicarb *vs*.

Maxiban showed that Nicarb produced significantly greater average weight, significantly improved feed conversion and significantly lower intestinal lesion scores than Maxiban.

A 38-day broiler chicken floor pen experiment was conducted to evaluate flock performance and confirm the results from the AST comparing Nicarb *vs*. Maxiban in the starter feed. The results showed that at both data collection periods (18 and 38 days) the birds supplemented with Nicarb had significantly better weight gain and feed conversion than the birds supplemented with Maxiban.

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Graph 1. Summary of anticoccidial sensitivity tests comparison between Nicarb vs. Maxiban for 26 mixed species isolates from 26 commercial broiler production complexes from various geographical regions of the United States.

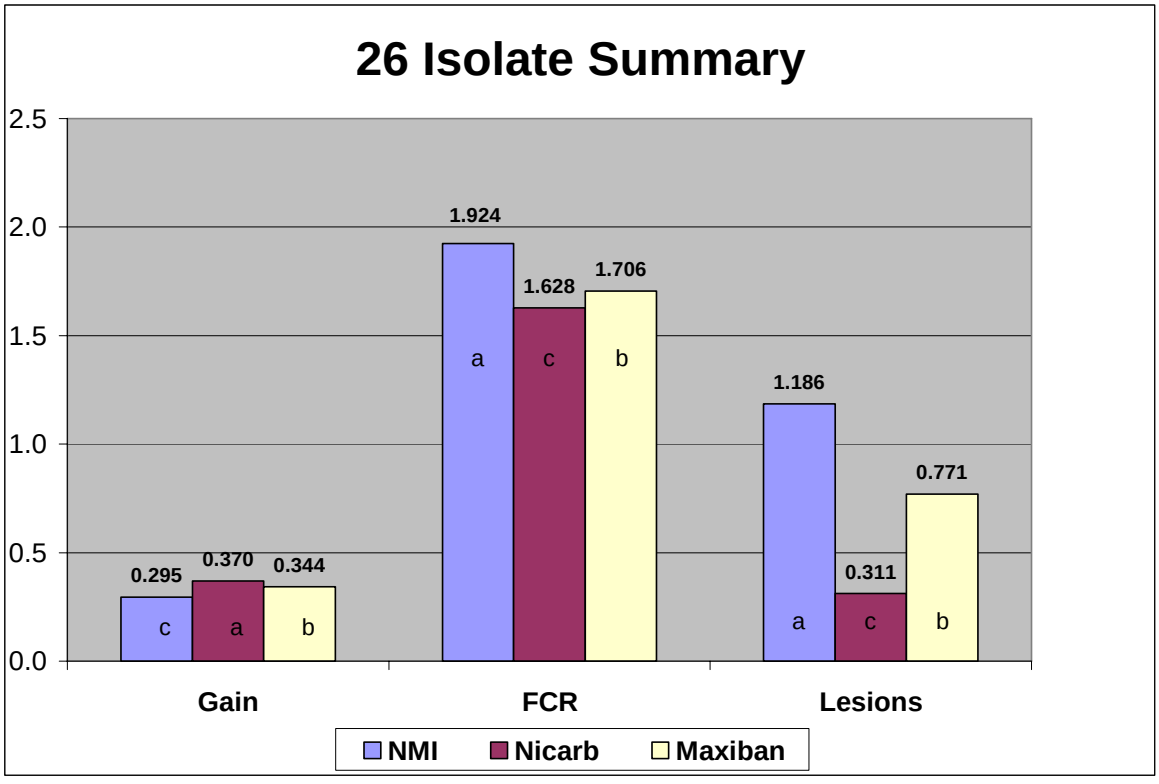

Table 1. Average body weight and feed conversion at 18 and 38 days of broiler chickens fed starter diets supplemented with either Nicarb (125 ppm) or Maxiban (79.2 ppm).

Parameter(s)	Nicarb	Maxiban
Body weight at 18 days (kg)	0.550[a]	0.506[b]
Feed conversion at 18 days	1.477[b]	1.564[a]
Body weight at 38 days (kg)	2.045[a]	1.997[b]
Feed conversion at 38 days	1.760[b]	1.797[a]

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

FIELD EXPERIENCES IN COMMERCIAL BROILERS WITH AN *IN OVO* COCCIDIOSIS VACCINE

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SUMMARY

Inovocox™ is a USDA licensed, sporulated oocyst vaccine composed of two non-attenuated strains of *Eimeria maxima* and one non-attenuated strain each of *Eimeria acervulina* and *Eimeria tenella*. Initial commercial usage began in the summer of 2006 with a commercial poultry integrator. The vaccine was administered *in ovo* at the time of transfer, with embryo age ranging from 18-19 days. It was injected into the embryonated egg in a volume of 0.050 mL Marek's vaccine diluent using the Embrex Inovoject® system, and was co-administered with Marek's disease and infectious bursal disease vaccines in the same *in ovo* injection. Hatchability, livability, clinical appearance, weight gain, feed conversion ratio, and condemnations were compared with broilers receiving the integrator's conventional coccidiosis control program.

INTRODUCTION

Coccidiosis control is essential for modern commercial poultry rearing. Since broiler *in ovo* vaccination is widely practiced today, particularly in the United States, for Marek's disease, infectious bursal disease, and other vaccines, it follows that an *in ovo* coccidiosis vaccine would be extremely advantageous. Inovocox is a USDA licensed, live oocyst coccidiosis vaccine developed by Embrex, Inc. specifically for *in ovo* administration. It is composed of two non-attenuated strains of *Eimeria maxima* and one non-attenuated strain each of *Eimeria acervulina* and *Eimeria tenella.* Previous studies have shown vaccination by live oocyst administration *in ovo* to be safe and efficacious with either manual or automated injection (1, 2, 3, 4). Further studies support the conclusion that *in ovo* coccidiosis vaccination does not interfere with Marek's vaccine efficacy (5) or with Bursaplex® (a live infectious bursal disease vaccine) efficacy (6). Other studies have shown Inovocox delivery through the Inovoject system is uniform in terms of numbers of oocysts delivered and also results in a high rate of vaccine infection early in the life of the birds (7, 8).

The objective of this paper is to relate comparative data obtained during the initial field usage of Inovocox under conventional industry production conditions.

MATERIALS AND METHODS

A 1X dose of Inovocox was administered to the eggs transferred during an entire week of production at one complex of a commercial poultry integrator, at either 18 or 19 days of incubation (E18 or E19). Inovocox was injected into the embryonated egg in a volume of 0.050 mL Marek's vaccine diluent using the Embrex Inovoject system, and was co-administered with the hatcheries standard complement of vaccines

(Marek's disease and infectious bursal disease) in the same *in ovo* injection.

Upon hatch, approximately 666,000 Inovocox-vaccinated chicks were placed on a total of 10 farms. The percent hatchability, percent livability, weight gain, feed conversion ratio, and total percentage of condemnations were compared with data from the week prior to the Inovocox placement week (referred to as control week 1) and the week after the Inovocox placement week (referred to as control week 2). Control week 1 placed approximately 795,000 chicks and control week 2 placed approximately 651,000 chicks. The control weeks were given the integrator's usual complement of *in ovo* vaccines and were treated with the integrator's conventional coccidiosis control program. With the exception of the coccidiosis vaccine, the vaccination program for the Inovocox and the control flocks was identical. All birds were raised on the same feed program and according to the management guidelines of the integrator. All Inovocox flocks were closely monitored by a trained poultry veterinarian who surveyed the chickens for general health, as well as any sign of clinical coccidiosis.

RESULTS AND DISCUSSION

There were no significant differences between the Inovocox treated flocks or the control flocks in hatchability, livability, weight gain, feed conversion ratio, or the total percentage of condemnations. Additionally, none of the Inovocox flocks exhibited any signs of clinical coccidiosis throughout the grow-out period. These data, along with other previously reported studies, indicate that the administration of Inovocox to broiler chickens via the Inovoject *in ovo* vaccination system provides safe and efficacious control of coccidiosis under conventional industry production conditions.

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CHARACTERIZATION AND ATTENUATION OF *EIMERIA MELEAGRIDIS* FROM THE TURKEY

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ABSTRACT

An isolate of *Eimeria* was obtained from the ceca of a turkey from northwest Arkansas and identified as *E. meleagridis* based upon the development of large schizonts in the mid-intestine, and small schizonts in the ceca. Two generations of large schizont were found 48 and 72 hr after infection, and at least two generations of small schizont from 60 to 108 hr after infection. An inoculum of 200,000 oocysts caused a significant reduction in weight gain after infection suggesting that the significance of this species as a pathogen of turkeys should be reassessed. A precocious line was developed by propagation of the parasite in turkey poults and collecting the first oocysts produced following infection. After 20 generations, the prepatent period was reduced by four to eight hours. Weight gain of poults given this line was significantly greater than that of poults given the parental strain indicating that selection resulted in a loss of pathogenicity. Poults immunized with the precocious line produced no oocysts following challenge with the parental strain indicating that immunogenicity was retained following selection. *E. meleagridis* is considered common worldwide and has been recorded in turkeys from twelve States in the USA. There may, therefore, be justification for including a precocious line of this species that has been attenuated by selection for early development in live vaccines for turkeys.

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

DOES AVIAN METAPNEUMOVIRUS (aMPV) EXACERBATE DISEASE CAUSED BY *RIEMERELLA ANATIPESTIFER* (RA) IN TURKEYS?

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INTRODUCTION

Riemerella anatipestifer (RA) is a Gram-negative bacterium of the family of *Flavobacteriaceae* (11). It is known to cause infectious septicemic and exudative diseases in a variety of bird species, including ducks, geese, wild waterfowl, and turkeys (4, 11).

Although reports of RA-outbreaks in turkey flocks associated with clinical disease and mortality are increasing (4), the pathogenicity of this bacterium for turkeys remains unclear. In a previous study we demonstrated that RA causes clinical disease and pathologic lesions in young turkey poults when administered via the respiratory tract (10). Gross pathology, such as fibrinous airsacculitis and adhesive pericarditis and epicarditis, was found to be of the same nature as reported from field outbreaks (4, 11). However, clinical signs and gross pathology were considerably less severe compared to experiences with RA from the field. This indicates that the presence of infectious and/or non-infectious factors may be necessary to exacerbate RA-induced lesions leading to the often severe disease observed in the field.

Avian metapneumovirus (aMPV), a member of *Paramyxoviridae*, is often isolated from diseased turkey flocks also positive for RA. aMPV induces an acute respiratory disease in turkeys, named turkey rhinotracheitis (TRT), causing considerable economical losses to the turkey industry (5). Four subtypes (A-D) of aMPV have been identified. Subtypes A and B are the dominating subtypes found in Europe (5).

aMPV is known to support secondary infections by causing lesions of the epithelium of the upper respiratory tract, such as ciliostasis, deciliation, and desquamation (5), as well as by induction of a systemic immunosuppression (3). Predisposing effects of aMPV on secondary bacterial infections in turkeys have been reported for *Ornithobacterium rhinotracheale*, *Bordetella avium*, *Escherichia coli*, and *Mycoplasma gallisepticum* (6, 8, 9, 12).

Therefore, infections with aMPV may also have predisposing and exacerbating effects on RA-infection of turkeys under field conditions.

In two experimental trials four-week-old commercial turkey poults were inoculated consecutively with aMPV subtype A or B and three to five days later with RA by aerosol. Dually infected animals showed higher rates of pathological lesions and RA-reisolation compared to groups receiving RA alone.

MATERIALS AND METHODS

RA and aMPV strains. A field isolate of RA serotype 1, isolated from a clinically diseased turkey flock in Germany, was used in this study. The aMPV subtype A strain BUT 8544 (aMPV-A) and a virulent subtype B strain from Italy (aMPV-B) were kindly provided by Dr. R.C. Jones (Liverpool, UK).

Reisolation of RA and detection of aMPV by RT-PCR. Tracheal swabs and organ samples were collected and streaked out on Columbia sheep blood (CSB) agar and incubated for 24 hours at microaerophil conditions. In the second experiment CSB agar containing 6 µg/mL neomycin sulphate and 20 µg/mL gentamycin sulphate was used in addition. This agar was found to be selective for RA. Genome of aMPV was detected from choanal swabs by nested RT-PCR (2).

Experiment 1. Thirty male commercial turkey poults were divided into three separately housed groups of 8, 11 and 11 birds at the age of 29 days. Group 3 (aMPV-B + RA 4) was inoculated oculonasally with aMPV-B (10^3 ciliostatic doses $(CD)_{50}$ per bird). Four days post viral infection (dpvi) animals of Group 2 (RA) and 3 were inoculated with RA by aerosol in an inhalation chamber. Each group was treated with an aerosol of 8 mL RA-solution containing 3.9×10^{11} colony forming units (CFU). Group 1 (Control) remained RA and aMPV free.

Clinical signs were recorded daily throughout the experiment. Tracheal swabs for RA-isolation were taken at 0 to 10, and 14 days post bacterial infection (dpbi). At days 0, 4, 7, 11, and 18 pvi, choanal swabs were collected for aMPV-detection. Turkeys were sacrificed and necropsied at days 3, 7, and 14 pbi, gross lesions were documented and samples of trachea, lung, air sacs, liver, spleen, heart, and brain were collected for RA-isolation.

Experiment 2. At the age of 32 days, 114 male commercial turkey poults were divided into five separately housed groups of 20 to 24 animals. Groups 2 (aMPV-A) and 5 (aMPV-A + RA 5) were inoculated with strain aMPV-A as described for Exp. 1. Two days later Group 4 (aMPV-A + RA 3) was inoculated with the same aMPV-strain. At day 5 pvi (representing day 3 pvi for Group 4) animals of Groups 3 (RA), 4 and 5 were infected with RA by aerosol. Subgroups of 12 animals received an aerosol of 8 mL RA-solution containing 1.1×10^{11} CFU. Group 1 (Control) remained RA and aMPV free.

Clinical signs were recorded daily throughout the experiment. Tracheal swabs for RA-isolation were taken at 0 to 10, 12 and 14 dpbi. Choanal swabs were collected at days 0, 5, 7, 10, and 14 pvi for aMPV-detection. Turkeys were sacrificed and necropsied at days 3, 6, 9, and 14 pbi, gross lesions were documented and organ samples for RA-isolation were collected similarly to Exp. 1.

RESULTS

Clinical signs. All groups infected with aMPV showed typical clinical signs, such as swollen infraorbital sinus and nasal and ocular discharge. Clinical signs peaked at days 6 and 7 pvi with no significant differences between aMPV-infected groups (data not shown). Turkeys infected with RA showed mild depression, huddling and ruffled feathers during the first three days after infection. No clinical signs were recorded from RA and aMPV-free control groups.

Gross pathology. Gross lesions were characterized by mild fibrinous serositis, such as airsacculitis, pericarditis and epicarditis. In Exp. 1 gross pathology was recorded in only four of 11 (36%) turkeys of Group 2 (RA) up to 14 days pbi, whereas eight of 11 (73%) turkeys of Group 3 (aMPV-B + RA 4) showed pathological lesions.

In Exp. 2 no differences between the RA-infected groups were evident. Rates of animals positive for gross pathology were 14/24 (58%), 14/24 (58%) and 12/23 (52%) for Groups 3 (RA), 4 (aMPV-A + RA 3) and 5 (aMPV-A + RA 5), respectively. No gross pathology was observed in any of the RA-free groups.

RA-reisolation from organ samples. RA was reisolated from at least one organ of all RA-infected animals of both experiments examined at day 3 pbi (Fig. 1). In Exp. 1 at this day 11 of 21 organs (52%) from Group 2 (RA) were RA-positive, compared to 16 of 21 (76%) positive organs from Group 3 (aMPV-B + RA 4). At day 7 pbi three of four turkeys of the dually infected Group 3 were RA-positive, whereas all

animals of Group 2 examined at this day were negative (Fig. 1A).

In Exp. 2 rates of RA-reisolation at 3 dpbi were 27 (64%), 32 (76%) and 31 (74%) out of 42 organs for Group 3 (RA), 4 (aMPV-A + RA 3) and 5 (aMPV-A + RA 5) respectively. At day 6 pbi one, two, and five out of six animals were found RA-positive in Group 3, 4 and 5, respectively (Fig. 1B). All RA-free animals and all turkeys necropsied later than day 7 pbi in both experiments were RA-negative (data not shown).

RA-reisolation from tracheal swabs. RA was reisolated from all swabs collected from RA-infected birds at days 1 to 3 pbi in both experiments. In Exp. 1 RA-reisolation in Group 2 (RA) thereafter declined completely until day 6 pbi, whereas rates of RA-reisolation of Group 3 (aMPV-B + RA 4) remained at high levels (25-100%) until day 10 pbi.

In Exp. 2 RA-reisolation declined in all RA-infected groups until day 6 pbi. No RA was isolated from groups 3 (RA) and 5 (aMPV-A + RA 5) later than day 6 pbi. RA-positive swabs were collected sporadically from Group 4 (aMPV-A + RA 3) until the end of the experiment at 14 dpbi. Swabs from RA-free groups remained negative for RA throughout both experiments.

aMPV-detection from choanal swabs. aMPV-genome was detected by RT-PCR from all aMPV-infected groups. aMPV-free groups were aMPV-negative at all tested time points.

DISCUSSION

In this study commercial turkey poults were infected with RA following aMPV-inoculation at different time intervals in comparison to turkeys receiving only RA. Animals of dually infected groups showed more prominent signs of disease, characterised by higher rates of gross lesions and RA-reisolation from tracheal swabs and organ samples. These differences were more pronounced in Exp. 1 when turkeys were infected with the aMPV subtype B strain compared to Exp. 2, in which a subtype A strain was used. Previous studies of our group have shown that the subtype B strain we used may cause more severe clinical disease and be more invasive than the subtype A strain (1, 7). This fact may have induced the slight variation between experiments of this study.

We conclude, that aMPV has a predisposing effect on RA-infection in turkeys and may exacerbate RA-induced diseases in the field. However, clinical signs and gross lesions found in the dually infected groups of this study were still relatively mild compared to field cases of RA-infections, indicating that management and environmental factors may contribute to the severity of these outbreaks in addition to predisposing viral infections.

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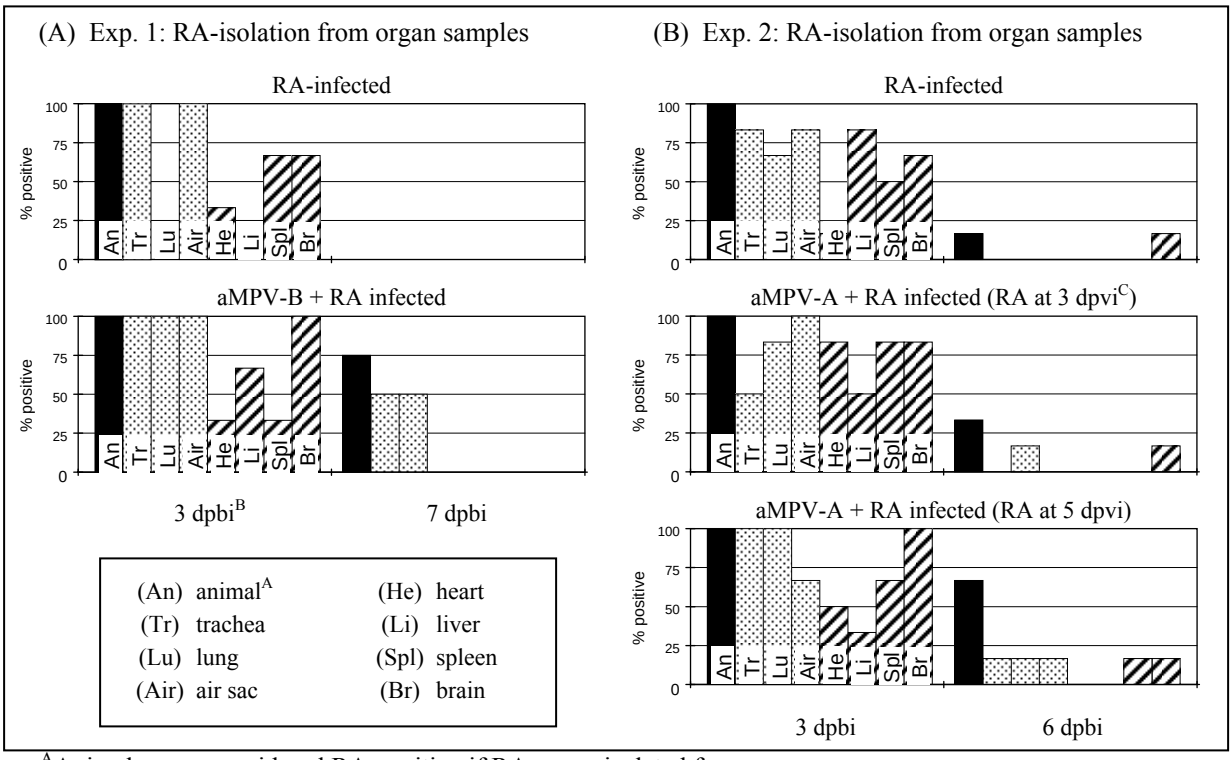
Figure 1. Reisolation of RA from organ samples collected at necropsy from RA-infected and aMPV + RA dually infected turkeys. (A) Exp.1: Dually infected turkeys were inoculated with aMPV-B and inoculated with RA at day 4 pvi (n = 3-4). (B) Exp. 2: Dually infected groups were inoculated with aMPV-A and inoculated with RA at day 3 or 5 pvi (n = 6).



[A]Animals were considered RA-positive if RA was reisolated from one or more organs.
[B]dpbi = days post bacterial infection. [C]dpvi = days post viral infection.

MOLECULAR DIAGNOSTIC OF AVIAN METAPNEUMOVIRUS (aMPV)

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Avian metapneumovirus (aMPV) causes an upper respiratory tract infection in turkeys leading to turkey rhinotracheitis, and in some other avian species including chickens, where it is also involved in the etiology of swollen head syndrome. In recent years, significant advances have been made in the diagnosis of aMPV based on PCR techniques (1-6). At the Minnesota Veterinary Diagnostic Laboratory (MVDL), an aMPV TaqMan® RT-PCR assay to detect copies of the matrix gene (M) was developed and has been used for the last six years. The purpose of our study was to improve the sensitivity of the TaqMan RT-PCR assay through nucleocapsid (N) gene targeting. We developed a TaqMan RT-PCR test using primers and probe specific for the N gene of aMPV subtype C. By optimizing the annealing temperature, primers and probe concentrations, and by making significant changes in the buffer composition, dye concentration as well as cycling conditions, we were able to develop an improved test with higher sensitivity and very good specificity compared to the original MDVL test. In fact, a side by side test comparison using both the traditional M-based TaqMan assay and the newly design N-based assay showed that the new test was at least 10 times more sensitive with the Colorado aMPV

isolate and up to 10,000 times more sensitive with the clinical and research samples submitted to the MDVL (Table1). The new assay was tested against several viruses and bacteria which include Newcastle disease virus, infectious bronchitis virus, avian influenza virus, paramyxoviruses 2 and 3, laryngotracheitis virus, *Pasteurella multocida*, *Escherichia coli*, *Bordetella avium*, *Ornithobacterium rhinotracheale*, *Mycoplasma* spp. and *Salmonella* spp. All tests were negative by the new N-based assay. All the results were confirmed by gel electrophoresis. Out of a total of 882 diagnostic samples including nasal swabs, choanal swabs, tracheal swabs, sinus swabs, tissues, and nasal turbinates, 226 samples were detected positive with the improved test versus only 98 samples with the old test. In addition of the higher sensitivity, the duration of the test was reduced from 4.5 hours to 1.33 hours and the cost was reduced by more that 40%. The current strategy used for the improvement of the aMPV diagnostic is being applied for the molecular diagnostic tests of other pathogens.

(The full-length article will be published in *J. Vet. Diagn. Invest.*)

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Table 1. Comparison between present APV-M TaqMan® assay and the newly designed APV-N TaqMan assay using clinical samples.

		APV-M			**APV-N**							
		Plus/Minus assay			**Plus/Minus assay**			**Real Time**				
		Call/Sample			Call/Sample			Call/Sample			CT	
Choanal swab	undiluted	+	+	+	+	+		+	+		30.65	
	10^{-1}	+	+	+	+	+		+	+		34.02	
	10^{-2}	+	-	-	+	+		+	+		36.23	
	10^{-3}	-	-	-	+	+		+	+		37.87	
	10^{-4}	-	-	-	+	+		+	+		38.78	
	10^{-5}	-	-	-	+	+		+	+		38.91	
	10^{-6}	-	-	-	+	-		+	-		Neg.	
	10^{-7}	-	-	-	-	-		-	-		Neg.	
	10^{-8}	-	-	-	-	-		-	-		Neg.	
	10^{-9}	-	-	-	-	-		-	-		Neg.	
Turbinate homogenate	undiluted	+	+	+	+	+	+	+	+		23.69	
	10^{-1}	+	+	+	+	+	+	+	+		26.35	
	10^{-2}	+	+	+	+	+	+	+	+		29.85	
	10^{-3}	+	+	+	+	+	+	+	+		33.35	
	10^{-4}	+	+	-	+	+	+	+	+	+	35.67	
	10^{-5}	-	-	-	+	+	+	+	+	+	38.01	
	10^{-6}	-	-	-	+	-	-	+	-	-	40.24	
	10^{-7}	-	-		-	-		-	-		Neg.	

COMPARATIVE EVALUATION OF DIFFERENT METHODS OF AVIAN METAPNEUMOVIRUS C VACCINATION IN TURKEYS

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ABSTRACT

Previous studies in our laboratory demonstrated that a single low volume spray avian metapneumovirus (aMPV) vaccination was not protective in turkeys against challenge. In the present study we evaluated seven different vaccination methods (eyedrop, spray cabinet (1x and 10x), low volume (1x and 10x) and high volume (1x and 10x) vaccinations) for their comparative efficacy. We evaluated immune response, reduction in clinical disease and virus shedding post challenge. Turkeys were challenged with aMPV; three weeks post first, second, and third vaccinations. Following the first vaccination challenge, birds in the low and high volume spray vaccinated groups showed clinical signs almost similar to the non-vaccinated but challenged control group whereas birds in the eyedrop and spray cabinet vaccinated groups showed very mild clinical signs post-challenge. However, birds in spray and eyedrop vaccinated challenged groups showed only minimal signs post second and third vaccine challenges. There was a significant increase in the GMT antibody scores of birds in the eyedrop and spray cabinet vaccinated groups upon challenge following first vaccination. Birds in the non-vaccinated, low and high volume spray vaccinated challenged groups showed significant virus shedding post first vaccine challenge whereas there was a considerable reduction in the virus shedding in the eyedrop and spray cabinet vaccinated groups. There was a significant reduction in the virus shedding in all the vaccinated challenged birds following second and third vaccinations.

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

ONSET OF IMMUNITY FOLLOWING AVIAN METAPNEUMOVIRUS (aMPV) VACCINATION *IN OVO*

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SUMMARY

Avian metapneumovirus (aMPV) was first reported in South Africa in the late 1970s. Since that time it has come to be recognized as a major disease of turkeys and chickens in many parts of the world. Three major subgroups, designated A, B, and C have been recognized since 1993, based on both sequencing of the G glycoprotein and neutralization tests with monoclonal antibodies. A fourth group, non A, non B (or subgroup D) is also recognized but is not thought to cause significant disease.

Vaccination of chickens and turkeys at day old by spray is well established and confers excellent protection against disease. However, as infection can occur early in life and spread of the virus throughout a flock is rapid, an early onset of immunity post vaccination could be beneficial to the poultry industry. We have studied the serological immune response and the onset of protective immunity of an aMPV vaccine delivered to chickens via the *in ovo* route in comparison to traditional vaccination via the oculonasal route at day old. Groups of twenty SPF chicken eggs were inoculated with different doses of the subtype B 11/94 based vaccine ($10^{4.5}$, $10^{3.5}$, $10^{2.5}$, or $10^{1.5}$ $TCID_{50}$) or with diluent. The chicks were allowed to hatch and were monitored for clinical signs. A further group of 20 non-vaccinated chicks were hatched and vaccinated via the oculonasal route with $10^{4.5}TCID_{50}$. All chicks were monitored for clinical signs of aMPV for 10 days. Six weeks post hatch all

chicks were bled, the serum collected and analyzed for aMPV-specific antibody responses by ELISA. The *in ovo* vaccinated chicks showed a significantly higher serological immune response than the birds vaccinated at day old.

To establish the onset of immunity in maternally derived positive chicks groups of 60 aMPV MDA positive chicken eggs were inoculated with graded doses of 11/94 vaccine ($10^{3.5}$, $10^{2.5}$, or $10^{1.5}$ $TCID_{50}$) or with diluent. Clinical signs of aMPV were monitored post hatch. A further group of 60 non-vaccinated chicks were hatched and vaccinated via the oculonasal route with $10^{1.5}TCID_{50}$. At 5, 8, 12, 15, 22, and 43 days post hatch groups of 8 to 10 vaccinated or control birds were bled for serum then challenged with virulent aMPV via the oculonasal route. Clinical signs of aMPV were monitored for 10 days post challenge. In the presence of maternally derived antibody (MDA), there was no significant difference in seroconversion between the vaccination routes though *in ovo* vaccination achieved a higher seroconversion rate. The onset of immunity (OOI) for the vaccine delivered to MDA positive chicken embryos was five days post hatch in *in ovo* vaccinated birds in comparison to eight days post hatch for the same dose of vaccine given at day old. The results demonstrate that an earlier onset of immunity can be achieved by *in ovo* vaccination in the face of MDA. Further work is required to establish the mechanisms responsible for this earlier onset of immunity.

(A full report of these studies has been submitted to *Avian Diseases*.)

HUMAN METAPNEUMOVIRUS CAUSES CLINICAL SIGNS IN TURKEYS

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SUMMARY

Human metapneumovirus (hMPV) and avian metapneumovirus (aMPV) belong to genus *Metapneumovirus* of *Paramyxoviridae* family. Neither virus is thought to cause cross-species infections. This experimental study was conducted to re-examine the hypothesis that hMPV will not infect turkeys. One hundred twenty two-week-old turkey poults were divided into six groups. Twenty poults in group 1 were inoculated oculonasally with 200µL of non-infected LLC-MK2 cell suspension. Twenty poults each in group 1 though 4 were inoculated with 200µL of one of the 4 genotypes of hMPV (A1, A2, B1, and B2). Birds in group 6 were inoculated with 200µL of aMPV subtype C oculonasally as controls. Birds inoculated with hMPV isolates showed clinical signs in the form of unilateral or bilateral watery to thick mucous nasal discharge from 4 to 9 days post-exposure (dpe). Specific viral RNA was detected in the nasal turbinates of birds exposed to each of the four genotypes of hMPV on 3 dpe by RT-PCR. Viral RNA was also detected on 5 dpe in one bird exposed to hMPV B2. Birds inoculated with hMPV were negative for aMPV RNA. Nasal turbinates of hMPV infected turkeys showed inflammatory changes and mucus accumulation. Immunohistochemistry revealed hMPV antigen in the apical surface of nasal turbinate in birds inoculated with hMPV. All four genotypes of hMPV caused a transient infection in turkeys as evidenced by clinical sign scores from 4 to 9 dpe, detection of hMPV RNA in the turbinate on 3 and 5 dpe, and histopathological lesions in the turbinate.

INTRODUCTION

Recent studies have shown that avian metapneumovirus (aMPV) subtype C isolates from domestic turkeys and wild birds in the USA show high sequence homology to hMPV (9, 13). Both the viruses belong to genus Metapneumovirus and share a projected amino acid identity of 56-88% (13). The aMPV is an upper respiratory tract pathogen of poultry with a global distribution (5). The USA was considered free of aMPV until an outbreak of respiratory tract infection in some turkey flocks in Colorado in May, 1996 (10). Later in 1997, the disease appeared in Minnesota, the largest turkey producing state in the country.

The disease caused by hMPV ranges from mild upper respiratory tract infection to severe bronchiolitis or bronchitis and pneumonia. The disease affects all age groups but young children, elderly, and immunocompromised individuals show severe disease manifestations (2). Serological surveys indicate that the virus is ubiquitous in nature and new infections can occur throughout life due to incomplete protection and genetic heterogeneity of the virus ((1). Clinically the disease is similar to that of human respiratory syncytial virus (hRSV) and after hRSV, hMPV is considered as one of the leading causes of bronchiolitis in young children (3). Though hMPV and aMPV are closely related to each other, they are not reported to cause cross-infection. A previous attempt to experimentally infect chickens and turkeys with hMPV was not successful (14). The objective of the present study was to re-examine the hypothesis that hMPV will not infect turkeys and for this purpose a preliminary experimental study was conducted by exposing two-week-old turkeys to four genotypes of hMPV.

MATERIALS AND METHODS

Cells and virus. LLC-MK2 cells (ATCC Number CCL-7) were maintained in Minimum Essential Medium (MEM, Invitrogen, Grand Island, NY) supplemented with 3% bovine fetal serum, 2mM L-glutamine, non-essential amino acids, 100 IU/mL of penicillin G sodium, and 100µg/mL streptomycin sulfate. Four genotypes of hMPV viz., A1 (GenBank Accession No: DQ312456), A2 (GenBank Accession No: DQ312449), B1 (GenBank Accession No: DQ312452), and B2 (GenBank Accession No: DQ312457) were used in the study. After the primary isolation these viruses were further propagated in LLC-MK2 cells in opti – MEM (Invitrogen) with 2µg/mL trypsin (replenished every other day), 100 IU/mL of Penicillin G sodium, 100µg/mL Streptomycin and no serum.

Avian MPV subtype C (aMPV C) was isolated from the nasal turbinates of 8-week-old turkeys with acute upper respiratory tract infection. The virus was passaged six times on chicken embryo fibroblasts (CEF) followed by six passages on Vero cells. The virus designated as aMPV/Minnesota/Turkey/19/2003 (aMPV MN 19) was used with a titer of 10^5 $TCID_{50}$ /mL.

Experimental design. One hundred twenty, two-week-old turkey poults were divided into 6 groups. Twenty poults in each group were inoculated oculonasally with 200µL of non-infected LLC-MK2 cell suspension (50 µL each in each eye and nostril), or one each of the 4 genotypes of hMPV (A1, A2, B1, B2), or aMPV C. Fresh untitrated virus propagated in LLC-MK2 cells were given to birds in the four treatment groups. To ascertain the amount of virus inoculated in birds in each group, each inoculum was later titrated by serial dilutions in LLC-MK2 cells. Birds were monitored daily for clinical signs. Two randomly selected birds from each group were euthanized at 3, 5, and 7 days post-exposure (dpe) for necropsy and sample collection. Nasal turbinate, trachea and lungs were tested to look for the presence of viral RNA by RT-PCR (6, 11). Tissue sections were stained by H & E and examined for histopathological lesions and for viral antigen by immunohistochemistry using hMPV and aMPV polyclonal antibodies (9). Sera collected from birds in each group at 14 and 21 dpe were tested using aMPV-ELISA (4).

RESULTS

Virus titer in each inoculum. The four genotypes of hMPV showed cytopathic effects in LLC-MK2 cells by 10-14 days post-inoculation. The hMPV genotype A1, A2, and B1 inocula had a titer of 10^3 $TCID_{50}$/mL each. Genotype B2 inoculum had a titer of 10^5 $TCID_{50}$/mL.

Clinical sign scoring. Birds inoculated with the four hMPV genotypes showed clinical signs in the form of unilateral or/and bilateral nasal discharge. The consistency of the discharge varied from watery to thick mucous. Clinical signs started on 4 dpe and stopped on 9 dpe. Birds infected with hMPV A1 recorded a clinical score range of 1 to 2 with 6/20 inoculated birds showing clinical signs. Birds infected with hMPV A2 had a score range of 1 to 8 with 12/20 birds showing clinical signs. In the case of hMPV B1, 7/20 inoculated birds showed clinical signs with a score range of 1 to 7. Among the groups, birds inoculated with hMPV B2 had the highest number of birds showing clinical signs (14/20) with a score range of 2 to 8.

Birds inoculated with aMPV MN 19 showed severe clinical signs with an average score of 14.17. The main signs were thick mucous bilateral nasal discharge and swelling of infraorbital sinus. No pathogenic bacterial colonies were isolated from nasal or tracheal swabs collected from7°C, in anaero birds exposed and non-exposed to one of the four genotypes of hMPV.

RT-PCR. The hMPV viral RNA was detected in the nasal turbinate of birds exposed to each of the four isolates of hMPV on 3 dpe by RT-PCR. Viral RNA was also detected on 5 dpe in one bird exposed to hMPV B2. Trachea and lungs did not show any viral RNA. Birds infected with aMPV showed viral RNA in nasal turbinate and trachea by aMPV specific RT-PCR on 3, 5, and 7 dpe.

Virus isolation. No cytopathic effects were detected in LLC-MK2 cell cultures inoculated with the

nasal turbinate homogenate from birds exposed to any one of the four genotypes of hMPV.

Histopathology. Nasal turbinates of turkey poults inoculated with all the four genotypes of hMPV showed mild to moderate histopathological lesions on 3 and 5 dpe. Inflammatory changes were more pronounced in birds inoculated with hMPV B2 than the other three genotypes. Lesions consisted of infiltration of inflammatory cells, mainly lymphocytes, macrophages and plasma cells, in the lamina propria. There was also accumulation of mucous in the nasal cavity and dilatation of mucous glands. Tracheas of birds inoculated with hMPV B2 showed mild inflammatory changes in the form of infiltration of inflammatory cells on 5 dpe. Nasal turbinate and trachea of birds infected with aMPV showed histopathological lesions in the form of infiltration of inflammatory cells, dilatation of mucosal glands and multifocal loss of cilia in turbinate.

Immunohistochemistry and Serology. The immunohistochemistry showed hMPV antigen in the epithelial surface of nasal turbinate of birds inoculated with all the genotypes of hMPV on 3 dpe. No antigen was detected in trachea or lungs. Birds infected with aMPV showed viral antigen in turbinate and trachea on 3, 5, and 7 dpe. No antibodies were detected in sera collected from birds exposed to hMPV on 14 and 21 dpe using aMPV-ELISA. However, aMPV infected birds were positive for aMPV-ELISA on 14 and 21 dpe There was no cross-reactivity between aMPV and hMPV in both the tests conducted using aMPV specific reagents whereas their respective controls with aMPV infected samples showed positive reactions.

DISCUSSION

In the present study, we investigated whether hMPV causes experimental infection in turkeys by exposing two-week-old turkeys to one of the four genotypes of hMPV, viz., A1, A2, B1, or B2. The results showed that all the four genotypes of hMPV infected turkeys as evidenced by clinical signs, RT-PCR results and histopathologic changes in the nasal turbinate and trachea of exposed turkeys. These findings are contradictory to those of van den Hoogen *et al*. (14) who did not detect any clinical signs or virus replication in juvenile turkeys inoculated with hMPV. The data presented in the above mentioned seminal paper on the retrospective discovery of hMPV from children in the Netherlands also indicated no experimental reproduction of infection in chickens (14).

In the present study, two-week-old turkeys were inoculated oculonasally with 2 X 10^2 $TCID_{50}$ of hMPV in three groups or 2 X 10^4 $TCID_{50}$ of hMPV in the fourth group. Birds receiving either dose developed clinical signs. In contrast, van den Hoogen *et al*. (14) inoculated 5 X 10^4 $TCID_{50}$ of hMPV in the conjunctivae and respiratory tracts of four juvenile turkeys and examined the birds for a three-week period for clinical signs and virus replication by sampling cloacal and throat swabs. Although van den Hoogen *et al*. (14) inoculated more virus than used in the present study, birds did not show clinical signs. So the amount of virus inoculated may not be the reason for the difference in results observed between the present and the aforementioned studies. The probable reasons for contradictory results could be origin of the virus, source, age and immune status of the turkeys used, and the samples that were analyzed. With aMPV infection, younger turkeys show more severe clinical signs than older turkeys (8, 15), and nasal turbinate, a major predilection site for aMPV replication in turkeys (15), is where we detected hMPV in this study.

The authors think that the differences in severity of clinical signs could be due to the difference in titer of these viruses inoculated in turkeys in this experiment. The amount of virus inoculated can potentially influence virus dissemination and pathogenicity. The virus inocula were titrated in LLC-MK2 cells after inoculating birds. The end point of titration was 14 days post-inoculation of the virus in cell cultures, and in order to use fresh inocula the authors decided to use untitrated virus. The main difference in clinical signs in birds infected with hMPV was the absence of infraorbital sinus swelling that is often associated with aMPV infection in turkeys (15).

The important question to ponder is the possible zoonotic potential of metapneumoviruses and their co-existence, if any, across the species barrier. Recent studies have shown that majority of infectious agents and the newly emerging pathogens in particular could be transmitted between humans and animals (12). Taylor *et al*. (12) reviewed literature on 1415 species of pathogens and identified 61% of them as zoonotic. Another interesting observation by the same authors was that 75% of the emerging pathogens they reviewed were zoonotic. In this context, the present findings necessitate further investigations on the zoonotic importance of hMPV.

The present study showed that all the four genotypes of hMPV caused a transient infection in turkeys as evidenced by clinical sign scores from 4-9 dpe, detection of hMPV RNA in nasal turbinate on 3 and 5 dpe, and histopathologic lesions in the turbinate and trachea. This is the first report of an experimental infection of turkeys with hMPV, and it opens up the possibilities of using turkeys as infection models for this virus. The present data suggest the need for a detailed investigation on the cross-species pathogenicity of hMPV and aMPV and their significance in human and animal health.

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THE SAFETY AND EFFICACY OF INJECTIBLE CEFTIOFUR IN BROILER BREEDERS

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SUMMARY

Excede™ was administered subcutaneously and intramuscularly to sex slip roosters to observe lethal effects and grossly observable tissue reactions. No mortality was seen during a five week period. Various tissue reactions were observed including mild ecchymotic lesions in the breast muscle and mild fatty infiltrations; however, all lesions were absent after five weeks. Two separate field efficacy trials were performed and were inconclusive.

INTRODUCTION

With the decreased access to available antibiotics, poultry veterinarians are left battling a number of pathogens with few options of treatment. In broiler breeders, for instance, few drugs can be used to combat *Staphylococcus* sp. and *Pasteurella* sp. in the breeding house. Ceftiofur is a viable option, as it is usually effective against both pathogens. Ceftiofur has been used in numerous hatcheries and is injected *in ovo* with other vaccines at transfer. Excede, a long-lasting form of ceftiofur, was tested in sex-slip roosters in the pullet house. The objective was to observe any lethal and/or grossly observable tissue reactions following intramuscular and subcutaneous injections of Excede with doses above the label dosage for swine.

MATERIALS AND METHODS

Thirty sex slip roosters were selected at 15 week handling. The dosage was calculated according to the label dosage (2.27 mg/lb). In an attempt to be above labeled dose for safety reasons with ensuing use, 0.13 mL was injected subcutaneously in the dorsal neck region and the same dosage injected intramuscularly in the left breast muscle. Each rooster used in the trial was painted for future identification and blended with the flock. Four roosters were euthanized and necropsied each week for the first 4 weeks. Twelve roosters were euthanized on the fifth week to finalize the trial prior to moving the flock to their designated hen farm.

Two separate field trials were performed in broiler breeder spike roosters. In the first trial, 150 spike roosters were penned in a breeder house for ten days before being released. The spike males experienced elevated mortality that was suspected to be caused by Staphylococcal infections. Mortality and clinical signs had subsided prior to release from the spike pen. In an attempt to ameliorate continuing Staphylococcal infections, at release, the roosters were injected with 0.2 mL of Excede intramuscularly.

In the second trial, 800 roosters were injected "metaphylactically" in the pullet house as they were loaded into live-haul cages to be moved to breeder houses in a different state. Even though the roosters were asymptomatic at the time of dosing, Excede was used in an attempt to control Staphylococcal synovitis. Upon arrival in the state of destination, these roosters were divided among five different breeder farms. The same route and dosage used in the first trial was also used in the second trial.

SAFETY RESULTS

Week 1 post-injection. Of the four roosters necropsied, three breast lesions were noted. Two of these breast lesions were mild ecchymotic lesions in the intermuscular, parasternal fascia of the left breast muscle, between superficial and deep pectoralis muscles. The other lesion noted was the same, with severe fatty infiltration in the same region. No subcutaneous neck lesions were noted.

Week 2 post-injection. Four roosters were necropsied. All four roosters had breast lesions similar to the lesions seen the first week. Three of the four lesions were mild ecchymotic hemorrhages, while the other only had fatty infiltration dispersed in the same area. Two birds also had 1 cm hemorrhagic lesions in the tissues surrounding the scapulohumeral joint. It was unclear whether this lesion was from the neck injection or an incidental finding.

Week 3 post-injection. Four roosters were necropsied. All four roosters had breast lesions. Two roosters had mild ecchymotic lesions as the previous weeks, while the other two were infiltrated with fat as seen in the second week. Hemorrhagic lesions were noted in three birds in the scapulohumeral region as was noted in the second week.

Week 4 post-injection. Four birds were necropsied. Only one breast lesion was noted, however it was severe and necrotic in the intermuscular,

parasternal fascia of the left breast muscle. Two birds had hemorrhagic lesions in the scapulohumeral region as in the second week.

Week 5 post-injection. Twelve roosters were necropsied to finalize the study. No breast lesions were noted in any of the roosters. The same shoulder lesions noted at week two were present in six of the twelve roosters. There were two unaccounted roosters left in the flock that were to be euthanized during the move to the hen farm.

EFFICACY RESULTS

The results from both trials were inconclusive. The first trial treated spike roosters that had recently cleared *Staphylococcus* infections and accompanying mortality. The second was a metaphylactic treatment of spike roosters. In both cases, no pathogen challenge was present at the time of injection. Mortality was assessed in both trials. (Table 1)

DISCUSSION

No fatal reactions were observed with the injection of Excede. Various lesions were seen at the injection sites; however, none were noted five weeks post-injection. Ecchymotic lesions noted were possibly due to the caustic nature of the solution. The necrotic lesion was possibly caused by a secondary infection, although no cultures were performed. It was difficult to attribute shoulder lesions to the subcutaneous injection of the drug.

The efficacy of Excede cannot be determined from the previously performed trials. To test antibiotic effectiveness, true pathogen challenge will need to be in place rather than a metaphylaxis-type injection. Once efficacy is determined, Excede may then be considered as a viable option in the control or prevention of various broiler breeder pathogens.

**Excede is approved for use in swine only. Extra-label usage is required if indicated in chickens.*

Table 1. Rooster mortality percentage post-injection of ceftiofur.

	Trial 1		Trial 2	
Week	**RR**	**SR**	**RR**	**SR**
1	1.48	11.33	0.86	2.88*
2	1.05	0.75*	1.19	6.82
3	2.42	0	1.21	5.52
4	-	-	0.87	4.97
5	-	-	0.74	3.38
6	-	-	0.96	1.59
7	-	-	0.96	1.94

*Released into Breeder House, RR-Resident Roosters, SR-Spike Roosters

EVALUATION OF BISMUTH SUBSALICYLATE FOR THE CONTROL OF NECROTIC ENTERITIS

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INTRODUCTION

Necrotic enteritis (NE) is an acute enterotoxemic condition of young chickens and turkeys, often associated with high sudden mortality for a short duration. The causative agent of necrotic enteritis is a Gram-positive obligate anaerobic bacteria *Clostridium perfringens* (CP), with mortality ranging from 2-50%. Signs of the disease are depression, ruffled feathers, and diarrhea. Gross lesions are usually found in the small intestine (jejunum) but have been seen in the large intestine and cecum. The classic lesion of NE is the intestinal mucosa covered with a tan to yellowish

pseudo membrane "Turkish towel" and having a characteristic foul odor.

During the early years of NE control, in-feed antimicrobials were the preferred products for the control of necrotic enteritis (NE). In recent years worldwide, there is pressure to remove or reduce the usage of in-feed antibiotics from poultry feeds. As a result, there has been a dramatic increase in the prevalence of necrotic enteritis in commercial poultry operations (5).

Anti-nutrients, such as the lectins from soybeans and wheat, cause damage to the epithelial tissues that aid in the attachment and proliferation of bacteria such as *Clostridium* spp. (5). Protease inhibitors found in heat-treated soybean meals may be present in varying amounts and negatively influence the digestibility of proteins (1). These higher protein levels increase the N concentration in the gastrointestinal tract (GIT) with subsequent overgrowth of CP in the intestine. Tannins are natural products of dietary ingredients such as rapeseed meals. These products may interact with proteins in the GIT and lead to tissue damage which may lead to NE (4, 10).

Several non-antibiotic methods of control of necrotic enteritis have been attempted. The use of enzymes such as xylanases and B-glucanases are used to aid in the break down of non-starch polysaccharides to increase the availability of digestible nutrients (3). Organic acids provide two possible methods of affecting the bacteria. Those acids with high pKa enhance the growth of non-pathogenic. Those with low pKa values are absorbed by the bacteria and eventually disrupt the biochemical pathways, leading to destruction of the organisms (2). Lactose in the diet has shown benefits, such as providing food source for *Lactobacillus* spp. growth and resultant reduction in pH of the ceca and *Clostridium* spp. growth (6, 9).

Insoluble salts of bismuth have been used as antimicrobial agents. A product containing bismuth subsalicylate (BSS) is used for the treatment of gastrointestinal disorders, including traveler's diarrhea. The proposed mode of action is a non-specific binding of bacteria to BSS and subsequent cessation of ATP synthesis or a loss of membrane integrity. The effectiveness is based on species and dose level of bacteria (8). The objective of this study is to evaluate the efficacy of BSS (Pepto-Bismol™) against NE using an NE disease model with or without coccidia.

MATERIAL AND METHODS

Experimental design. Day old commercial broiler chickens were assigned to eight treatment (trt) groups in separate floor pens with wood shavings: *E. maxima* (EM) alone (trt 1), *C. perfringens* (CP) alone (trt 2), EM plus CP (trt 3), EM plus 1 mL oral gavage of Pepto-Bismol™ extra strength (PB) (trt 4), CP plus PB (trt 5), *E. maxima* plus CP plus PB (trt 6), PB only (trt 7) negative control (trt 8). There were 25 birds per pen. All birds were fed a non-medicated commercial basal ration (Southern states™) of 17% crude protein from 1-26 days of age. Trt 1 and 3 were orally gavaged with 1 mL of inoculum containing 50,000 EM oocysts /mL at 22 days of age. At 26 days, the feed was withheld for 4 hr. It was replaced with a different commercial non-medicated feed containing 24% crude protein (Dumor™ starter). Birds in trt 4-7 were orally gavaged with 1mL PB using a syringe and a flexible plastic tubing. A CP inoculum was prepared using cooked meat broth incubated at 3bic jars using the Mitsubishi AnaeroPak® system. The CP titer is approximately 7-8 log colony forming units (CFU)/mL. Birds in trt 2, 3, 5, and 6 were orally gavaged with 4 mL of the CP inoculum 1 hr after PB administration. Six birds from all treatments and the control group were humanely euthanatized for postmortem evaluation and sample collection each day from day 1-3 CP post-inoculation.

RESULTS AND DISCUSSION

After 24 hr CP post-inoculation three birds in trt 3 (EM plus CP) had died due to acute enterotoxemia and showing severe NE classical signs and lesions. Within days 1-3 CP post-inoculation, infected birds in trt 4-6 given 1mL PB had no gross or microscopic lesions of suggestive of necrotic enteritis. However, birds that did not receive PB had gross and microscopic lesions consistent with severe EM coccidiosis (trt 1, 3, 4) and/or NE (trt 2, 3) and consequent mortality due to severe NE. Birds that had received EM only plus PB had gross and microscopic pathology consistent with EM coccidiosis. Birds treated with PB only (trt 7) have gut morphology (gross and microscopic) indistinguish-able from the negative control (trt 8). Histopathological findings were diffused, multifocal or focal necrotic enteritis lesions. These were similar to those of birds having NE but with concomitant coccidial infections. NE appeared to begin at the tips of the villi and spread to the upper half of the mucosal surface. At this dose regimen, PB has demonstrated effectiveness in controlling NE but has no inhibitory effect on EM coccidiosis.

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COMPARISON OF BIO-MOS® AND BACITRACIN AS GROWTH PROMOTERS IN BROILERS AND TURKEYS

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ABSTRACT

Antibiotic growth promoters have been a standard inclusion in meat bird rations for many years. To maintain their effectiveness, it has been found best to shuttle or rotate them. Recent events have decreased the numbers of available antibiotic growth promoters and the chances of new antibiotics for use as growth promoters are remote. Bio-Mos (yeast derived glucomannan) provides comparable growth promotion to bacitracin. Bio-Mos has a different mode of action to antibiotics, however, and thus will fit well in either a shuttle or rotation program, giving an added option to current antibiotics.

INTRODUCTION

Antibiotic growth promoters (AGP) have been a standard part of many turkey and broiler feeding programs since the 1940s (1). Recently, their use has been questioned and legislative action has been taken in the European Community to reduce and eliminate their use. In North America there has been selective action taken against their use at growth promoting levels. Many integrators have, however, voluntarily reduced or eliminated the use of AGPs. These actions have been taken for economic and marketing reasons. Action against AGPs is, in part, based on the increased levels of resistant genes that protect pathogenic bacteria. These resistant genes may be both specific and/or more general in nature. The cause of resistant genes is not completely understood. There are those that feel that development of resistance in the bacterial microflora of poultry and livestock has not been proven to transfer to humans. There is good reason to believe that resistance can develop in human gut microflora separate from farm animals. It is clear that: a) poultry fed AGPs develop resistance that causes the effectiveness of that specific AGP to decrease thereby requiring the shuttling or rotation of AGPs, and b) some consumers of poultry meat want the use of AGPs reduced or eliminated.

Bio-Mos (2) has demonstrated the ability to effectively inhibit conjugation *in vitro,* decreasing the spread of antibiotic resistance transfer among *E. coli,* thus providing a strategy to control multi-drug resistance. Bio-Mos gave comparable body weight gain and feed conversion in both broilers (3) and turkeys (4) with significantly improved livability when compared to rations containing AGPs. Thus, Bio-Mos can be used in growth promotion programs in place of AGPs with comparable performance. When used in the starter and grower phases it would reduce the prevalence of

antibiotic resistance and thus enhance the effectiveness of AGPs used in the finisher phase.

RESULTS AND CONCLUSIONS

Trials with both broilers and turkeys are summarized in the accompanying table. Body weight, feed conversion, and mortality did not differ statistically between treatments in any of the trials, nor was one treatment consistently better numerically than the other. It can be concluded, therefore, that Bio-Mos and bacitracin are comparable when used for growth promotion. This conclusion is similar to (12) that found using halo analysis when it was concluded that 1 g/kg of Bio-Mos was comparable to 50 ppm of bacitracin.

Bio-Mos can replace bacitracin in a shuttle or rotation growth program with no loss in performance. It will enhance the performance of the AGP that it is used with because of its action against the spread of resistant genes.

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Table1. Comparison of Bio-Mos with bacitracin in trial with both broilers and turkeys.

Age (days)	Body wt. (kg)		Feed conversion		Mortality (%)		Reference
	BM[1]	BAC[2]	BM	BAC	BM	BAC	
Broilers							
49	2.505	2.578	1.830	1.815	4.58	5.42	5
35	1.915	1.094	1.840	1.860	--	--	6
42	1.894	1.839	1.849	1.766	--	--	7
Turkeys							
98	7.612	7.655	2.196	2.244	2.22	5.19	8
126	12.563	12.455	3.122	3.154	15.63	14.40	9
140	13.299	13.118	2.539	2.554	6.67	14.00	10
147	17.631	17.418	2.520	2.520	8.76	8.61	11

[1] BM = Bio-Mos
[2]BAC = bacitracin

ASSESSMENT OF ALBENDAZOLE FOR THE TREATMENT OF COMMON CHICKEN HELMINTHS

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ABSTRACT

Sixty naturally infected hens obtained from a broiler-breeder farm in Northwest Arkansas were used in a controlled titration study to determine the anthelmintic efficacy of albendazole (VALBAZEN® Pfizer) in the treatment of both nematode and cestode infections. Albendazole was used at the dose rates of 0.0, 5.0, 10.0, and 20.0 mg/kg BW, hence there were 15 birds per treatment group. All treatments given as an oral suspension on day 0 (split doses) and the birds were necropsied for parasite collection on day seven. Statistically significant ($P < 0.05$) reductions in worm burdens from control levels were accomplished at the 5.0 mg/kg dose level for adult and larval stages of *Ascaridia galli, Heterakis gallinarum*, and *Capillaria obsignata*. Significant ($P<0.05$) reduction in the numbers of *Raillietina cesticillus* (scolexes) from control group levels was seen only at the 20.0 mg/kg rate of treatment. For albendazole given at the rates of 5.0, 10.0, and 20.0 mg/kg, respectively, anthelmintic efficacies were 87.7, 91.2, and 98.2% for *A galli* larvae; 100.0, 100.0, and 100.0% for *A galli* adults; 96.9, 95.7, and 98.9% for *H gallinarum* larvae; 92.7, 95.4, and 94.9% for *H gallinarum* adults; 90.3, 91.3, and 95.1% for *C obsignata* larvae and adults combined; and 73.1, 73.1, and 96.2% for *R cesticillus*. No adverse reactions to albendazole were noted in this study.

COMPARISON OF ELISA AND AGID TESTS FOR THE DETECTION OF FOWL ADENOVIRUS SPECIFIC ANTIBODIES

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SUMMARY

The number of cases of clinical diseases, such as inclusion body hepatitis (IBH) associated with fowl adenoviruses (FAdV) is increasing. IBH is a concern in different parts of the world including North America, and it became one of the major diseases of broilers in Canada. In addition to virus detection and characterization, flock monitoring by serological methods is becoming an important component of IBH management. There are different methods available for FAdV specific antibody (Ab) detection. The agar gel immunodiffusion test (AGID) is a routinely used test in most, if not all, diagnostic laboratories. Earlier we described an FAdV specific group-cross reactive enzyme-linked immunosorbent assay (ELISA) for FAdV Abs which is based on FAdV-9 as antigen. The purpose of this project was to evaluate this ELISA on field samples and compare it to the AGID test. Additionally, the performance of FAdV-9 coated ELISA plates was compared to that of FAdV-8-antigen based ELISA.

Altogether, 1118 serum samples originating from 84 broiler and breeder flocks and collected in four provinces of Canada were tested. At the individual level 41.6% of the samples were positive by AGID, 9.7% of them were suspicious and 48.7% were negative; by FAdV-8 based antigen ELISA 76.5% were positive, 2.3% were suspicious and 21.2% were negative; and by FAdV-9 based ELISA 85.3% were positive, 2.9% suspicious and 11.8% negative. At the flock level, based on the AGID test 69% of the flocks were positive and 31% of them were negative. On the other hand, 87% and 95% of the flocks were positive by the FAdV-8 and FAdV-9 ELISA, respectively.

These data indicated that our ELISA is more sensitive than the AGID test, and the performance of the FAdV-9 antigen ELISA is superior to that of the FAdV-8 antigen based ELISA.

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

INCLUSION BODY HEPATITIS OF CHICKENS – OCCURRENCE AND CONTROL IN AUSTRALIA

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SUMMARY

Avian adenoviruses (AAV) have been isolated from poultry in Australia since the 1960s, but inclusion body hepatitis (IBH) outbreaks from which AAV were isolated were not reported until 1974. Early outbreaks of IBH were considered to be precipitated by immunosuppression, particularly due to infectious bursal disease (IBD) virus. However, in the early 1980s an acute form of IBH occurred in broilers throughout Australia characterized by mortality rates as high as 40% over a ten-day period commencing at 7-14 days of age. Outbreaks occurred for four to six weeks in most progeny broiler flocks from specific breeding flocks. A serotype-8 (now 8b) AAV was isolated from livers of affected birds. Intranuclear basophilic inclusion bodies predominated in the livers of affected birds. IBH was

reproduced with a serotype-8 AAV by inoculating day-old specific-pathogen-free (SPF) chickens by natural routes of infection and IBH occurred in in-contact chickens. Virological and serological evidence indicated that IBD virus or chicken anemia virus (CAV) was not involved. A live vaccine, developed using one of the virulent serotype-8 AAV isolates and used to vaccinate breeders during rearing to ensure seroconversion prior to onset of lay, has been an integral part of a strategy to successfully control IBH in chickens in Australia in the last 15 years.

INTRODUCTION

The most important disease of chickens in which AAV have been involved is IBH, with early reports in the USA (19), Canada (21, 28) and the United Kingdom (24, 43). A serotype-5 AAV, designated Tipton strain, was the first AAV incriminated in the etiology of IBH (6, 11, 35). Type-8 (14, 16) and other serotypes of AAV (25) have also been isolated from IBH outbreaks. The pathogenesis of IBH was considered to be primary immunosuppression caused by IBD virus, followed by AAV infection (12, 36). Aplastic anemia was sometimes linked with AAV and IBH, but CAV may have been involved in these cases (7). Hydropericardium Syndrome or Angara Disease, that initially was reported in Pakistan (2) and then in a number of other countries, is a variation of IBH occurring at three to six weeks of age and with serotype-4 AAV being involved (9,20).

IBH OUTBREAKS IN AUSTRALIA

The earliest reports of IBH in Australia were reported in 1974 based on histopathological lesions, the isolation of an AAV in a 1973 outbreak, and reproduction of characteristic lesions by intravenous and intraperitoneal inoculation of seven-day old chickens (41, 42). In the most severe outbreak mortality rates between 8.4% and 32.3%, commencing at 35-40 days and extending to 70 days, occurred on five broiler farms of one poultry company. Atrophy of the bursa of Fabricius, anemia, cellulitis, eosinophilic Cowdry-type intranuclear inclusions, and secondary bacterial infections occurred in these cases. There were other early reports of IBH in Australia (4, 23, 30, 31). Serotype 8 (22) and serotypes 6, 7, 8, 6/7/8 and untypable (23, 32) were isolated from affected livers containing either eosinophilic or basophilic intranuclear inclusions. Because there were various serotypes isolated, the outbreaks occurred in birds older than 21 days, and there was atrophy of immune system organs, it was considered at the time that some other agent, for example IBD virus or in retrospect CAV, was the primary cause of IBH in Australia with AAV being secondary opportunistic pathogens. Potent inactivated IBD vaccines only became available in Australia in 1987, mainly due to the awareness that intermediate-plus types of IBD viruses that occur in Australia can be immunosuppressive and could contribute to IBH outbreaks. It wasn't until 1995 that a live virulent CAV vaccine, similar to that developed in Germany (40) and still sold worldwide today, was available in Australia.

The possibility that AAV could be a primary pathogen in IBH outbreaks was postulated when a type 2/12 AAV was diagnosed in IBH in a 12-week old, specific-pathogen-free (SPF) cockerel (33). While there were previous reports that AAV could be vertically transmitted (25), it was not considered that this led to IBH occurrence. IBH outbreaks causing up to 40% mortality over a ten-day period commencing in flocks at 7-14 days of age with a predominance of serotype-8 AAV and the finding that some affected flocks had no detectable maternal antibody to IBD virus (32) provided circumstantial evidence that AAV could be the primary pathogen. Outbreaks occurred mainly in poultry companies that had superior biosecurity and sanitation programs in their breeder operations. Experimental reproduction of IBH in day-old SPF chickens dosed orally with a serotype-8 AAV and the occurrence of IBH in in-contact chicks provided further evidence that AAV could be a primary pathogen in IBH outbreaks (5, 10, 34). A feature of these "acute" IBH outbreaks was that basophilic inclusions predominated over eosinophilic inclusions (32) which may be the result of the rapid onset of acute IBH and death shortly after infection (15,34). Involvement of IBD virus, CAV, and reticuloendotheliosis virus seemed unlikely on the basis that convalescent sera were negative to these viruses (5, 10).

AAV shown to have different degrees of pathogenicity were grouped into molecular Group E (contains serotypes 6, 7, 8a and 8b), but the AAV isolated from acute cases of IBH in flocks under three weeks of age were predominantly serotype-8 AAV, were the most virulent in challenge studies, and could be differentiated from the remaining Group E AAV on pairwise comigrating restriction fragment (PCRF) analysis (10). Further studies in which recombinant AAV were developed indicated that the fiber was responsible for differences in virulence (27).

Epidemiologic evidence during an outbreak of IBH in New Zealand also suggested that IBH could result from vertical transmission of AAV (8), although the outbreaks commenced between three and four weeks of age and thymic atrophy and anemia occurred in affected broilers. Thymic and bursal atrophy occurred when a serotype-8 AAV isolated in NZ produced acute IBH when administered by natural

routes of infection (37, 38). IBD virus could not have been involved in the NZ outbreaks, as NZ chicken flocks were negative to this virus at the time. Epidemiological experience within an Australian national integrated chicken meat company also led to the conclusion that outbreaks of acute IBH due to serotype-8 AAV in flocks less than 14 days of age were vertically transmitted (E. Arzey, NSW Department of Primary Industries, personal communication, 17). There was a correlation between acute IBH outbreaks due to serotype-8 AAV in 52 flocks and specific breeder flocks. Seroconversion to serotype-8 AAV was demonstrated in two grandparent flocks which produced parent flocks that were placed over a six week period in different states and had outbreaks of acute IBH. Similar seroconversion correlations were found between parent flocks and broiler progeny with acute IBH. Clinical signs of runting and paleness, low blood packed cell volumes, autopsy lesions of pale bone marrow and thymic atrophy, and secondary infections with *Escherichia coli*, *Salmonella*, and aspergillosis, which are commonly seen in CAV-related disease, were not common features of acute IBH. IBH outbreaks have also been thought to have been due to a vertically transmitted serotype-3 AAV in the USA (29) and serotypes 7, 8 and 11 in Canada (18). Maternal antibody was protective when day-old SPF chicks were injected intra-abdominally with a serotype-8 AAV (13). Protection studies in Australia raised the possibility that a serotype-8 AAV vaccine could be developed to prevent acute cases of IBH (26).

CONTROL OF IBH IN AUSTRALIA

Because serotype-8 AAV were isolated from the majority of outbreaks of acute IBH that occurred in chickens less than 14 days of age throughout Australia, there was evidence of vertical transmission, the disease had been reproduced in SPF chickens by natural routes of administration with plaque-purified serotype-8 AAV, the involvement of common immunosuppressant viruses had been eliminated, and there was evidence that live serotype-8 AAV were protective against homologous challenge, a decision was made in 1989 to progress the development of a live serotype-8 AAV vaccine for use in breeders to prevent "acute" IBH in progeny.

A virulent serotype-8 AAV, now classified as 8b by the International Committee on Taxonomy of Viruses (3), isolated in chicken embryo liver cell cultures from the livers of nine-day-old meat chickens with acute IBH was purified by limit-dilution technique, seedstocks produced in SPF cell cultures and an AAV vaccine was manufactured. Replacement breeders vaccinated *per os* or by drinking water administration to infect at least 20% of the flock in mid rearing were bled at 16 weeks of age and tested for serum neutralizing antibodies against serotype-8 AAV to confirm successful vaccination of the flock. This vaccination approach, administering a vaccine containing a virulent virus to a portion of a breeder flock during rearing, had been used successfully for many years to control avian encephalomyelitis in Australia and a similar approach had been used in Germany to control CAV disease (40).

Vaccination of all meat and layer breeders of one national poultry company in Australia with this vaccine containing serotype 8 (now 8b) AAV commenced in 1990 and all other Australian chicken meat breeder companies adopted a similar vaccination program when the vaccine (Intervet Australia FAV vaccine) became available. Field experience over the last 15 years has indicated that the vaccine is safe, breeding flocks become serologically positive to serotype-8 AAV prior to onset of lay, and acute IBH outbreaks have not occurred in progeny adequately immunized as judged from serological response. In fact, this vaccination approach has been regarded in Australia as a great "success story" for the control of an economically important poultry disease.

However there have been sporadic occurrences of IBH in Australian broiler flocks in the last year. Low mortality has occurred at 10-35 days of age. AAV isolated from only two outbreaks were typed (Intervet Boxmeer) being 2/12 in one outbreak and 9 with 8/6 cross reactions in the other. Investigations by technical staff of the poultry companies involved indicated that immunosuppression may have been the primary cause of these outbreaks. Because AAV serotyping is not currently available in Australia, funding for a research project has been made available to the University of Melbourne by the Rural Industries Research and Development Corporation to facilitate appropriate investigations into any future IBH outbreaks. If there were widespread acute IBH outbreaks due to a specific AAV serotype or biotype other than that already included in the vaccine, then the need to include that AAV in the current live AAV vaccine would have to be considered. A similar approach has been reported with the use of inactivated adenovirus vaccines in some countries to prevent IBH (1,9) and hydropericardium syndrome (25). Live vaccines are usually less expensive and administration costs are lower than for inactivated vaccines.

DISCUSSION

While it is accepted that immunosuppressive viruses can facilitate various AAV serotypes to cause IBH, there is also adequate evidence that some AAV can cause an acute form of IBH via vertical

transmission. However it is sometimes difficult to determine the exact epidemiology of specific IBH outbreaks. Experimental reproduction of IBH using natural routes of infection usually results in IBH within three to five days and cannot usually be achieved in susceptible chicks older than about 10 of age (34), even when AAV are injected (11,15), unless birds are immunocompromised (34). Hence it is difficult to explain how IBH/hydropericardium outbreaks that commence in flocks older than four weeks of age are due to primary AAV infection or how vaccination of breeders with AAV vaccines can modify such disease occurrences. It may be that some virulent AAV can be the primary "immunosuppressants", since AAV have been shown experimentally to cause atrophy of the bursa of Fabricius and the thymus (37,38). This would permit AAV to cause IBH in birds older than those in which IBH can be reproduced experimentally by natural routes of infection and could justify the use of vaccines, whether live or killed, in breeders to prevent IBH outbreaks in flocks as old as three to five weeks. Hence control of IBH in Australia has been by adopting appropriate vaccination programs for the immunosuppressants CAV, Marek's disease virus, and IBD virus and by vaccinating breeding flocks with a live AAV vaccine which contains a serotype-8 (now 8b) AAV (Intervet Australia FAV vaccine).

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EXPERIMENTAL STUDIES INDICATE AN ETIOLOGIC ROLE FOR ADENOVIRUS-LIKE VIRUS (ISOLATE R11/3) IN TRANSMISSIBLE VIRAL PROVENTRICULITIS

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Transmissible viral proventriculitis (TVP) is a poorly understood disease of broiler chickens that is characterized by proventricular enlargement, degeneration and necrosis of proventricular glandular epithelium, ductal epithelial hyperplasia, and lymphocytic inflammation. The disease occurs commonly in broiler chickens and is associated with proventricular fragility, impaired growth ("runting"), poor feed conversion, and impaired feed digestion. TVP also is responsible for increased processing costs due to higher numbers of reprocessed carcasses, downgrades, and condemnations. In a recent study, an adenovirus-like virus (AdLV), designated AdLV (R11/3), was isolated from proventriculi obtained from TVP-affected broiler chickens; the virus was determined to have morphologic and biologic characteristics consistent with adenoviruses, but antigenic and genomic characterization studies indicated that the virus was distinct from known avian adenoviruses.

TVP was experimentally reproduced in two-week-old specific-pathogen-free chickens and commercial broiler chickens by eye drop inoculation of AdLV (R11/3). No clinical signs and no weight gain depression were observed in chickens inoculated with AdLV (R11/3); however, gross and microscopic lesions characteristic of TVP were present in proventriculi of inoculated chickens. Enlargement of proventriculi of AdLV (R11/3)-inoculated chickens, compared with sham-inoculated controls, was evident by day 7 post inoculation (PI). Microscopic lesions characteristic of TVP were detected in proventriculi of inoculated chickens beginning on day 3 PI; no microscopic lesions were observed in other tissues. AdLV (R11/3) antigens were detected in proventriculi by immunohistochemistry on days 3–10 PI in inoculated SPF chickens and days 3-21 PI in inoculated commercial broiler chickens; no viral antigens were detected in other tissues. AdLV (R11/3) was reisolated from proventriculi of inoculated SPF and commercial broiler chickens on days 5 and 7 PI. No virus, viral antigens or lesions were detected in proventriculi collected from sham-inoculated chickens. These findings indicate an etiologic role for AdLV (R11/3) in TVP.

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EFFECTS OF INFECTIOUS BRONCHITIS VIRUS AND *ESCHERICHIA COLI* CO-INFECTION ON THE PATHOGENESIS OF *ORNITHOBACTERIUM RHINOTRACHEALE* IN LAYING CHICKENS

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SUMMARY

Ornithobacterium rhinotracheale (ORT) causes respiratory infection in turkeys and chickens. The respiratory infection results in airsaculitis and pneumonia. These lesions can be very severe when turkeys and chickens have concurrent infections with other respiratory pathogens. The objectives of this study were to investigate the pathogenesis of ORT infection alone and with exposure to infections with infectious bronchitis virus (IBV) and *Escherichia coli* in laying chickens. Briefly, one hundred, seventy two-week-old SPF white leg-horn chickens, free of ORT infection were exposed experimentally to either ORT and IBV alone or with ORT+IBV, IBV+*E. coli*, and ORT+IBV+*E. coli* combinations. The clinical signs and pathological changes in the internal organs were evaluated in birds in all groups. Birds infected with ORT alone did not show any visible clinical signs but

birds infected with IBV, ORT+IBV, IBV+*E. coli*, and ORT+IBV+*E. coli* showed clinical signs like ruffled feathers, droopiness, and nasal discharge. Birds showed gross lesions during necropsy such as hemorrhagic tracheitis, fibrinous pneumonia, airsaculitis, pericarditis, and peritonitis. Histopathological studies revealed marked pathological changes in the tissues examined. Mortality was noticed only in the ORT+IBV+*E. coli* co-infected group with a maximum mortality of 10% in 60 days. Our results suggest that IBV and *E. coli* co-infection aggravates ORT pathogenesis in adult laying chickens. It also showed that ORT could cause both acute and chronic infections when co-infected with IBV and *E. coli* in laying chickens.

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a Gram-negative rod-shaped bacterium that has been associated with outbreaks of respiratory disease in turkeys and chickens (3). It causes decreased feed intake, growth retardation, reduced egg production, mortality, and increased rate of slaughter condemnation in poultry (8). In turkeys, ORT causes severe respiratory lesions like tracheitis, airsacculitis, fibrinous pneumonia, and pericarditis (6, 8).

The first report of ORT infection in US turkeys came in the year 1993 from California (3), and four years later isolation of ORT from turkeys was documented in Minnesota (6). In layer chickens, ORT infection in the US was first reported in 2000 from Minnesota where layer flocks were affected with pericarditis, airsacculitis, and peritonitis (7). But along with ORT, other pathogenic agents such as infectious bronchitis virus (IBV) and *Escherichia coli* were also isolated and ORT was believed to be the inciting cause of that severe outbreak. The seroprevalence of ORT in chickens was reported to be as high as 100% in broiler breeder flocks in Southern Brazil (2) and in layer flocks in North central regions of United States (5). In chickens, even though the seroprevalence is very high, there are not many reports of isolation of ORT or cases of severe outbreaks or high mortality as do occur in turkeys. The clinical disease caused by ORT in chickens is less severe than in turkeys and involves sneezing, rhinitis, and facial edema. In many outbreaks and experimental infections in chickens, ORT did not produce clinical signs or mortality, but lesions were produced in the localized regions of the respiratory tract. Most of the severe respiratory lesions like airsacculitis and pneumonia in chickens were reported to be caused by a concomitant ORT infection with other respiratory pathogens.

The objectives of this study were to investigate the role of ORT in the pathogenesis of respiratory disease caused by ORT alone or with concomitant infectious bronchitis infection in laying chickens and to investigate the role of ORT in the pathogenesis of respiratory disease caused by concomitant IBV and *E. coli* infection in laying chickens.

MATERIALS AND METHODS

Ornithobacterium rhinotracheale (strain 31C) was grown on 5% sheep blood agar (SBA) containing antibiotic gentamycin (10µg/mL) in 5% carbon dioxide at 37°C for 48 h. *E. coli* serotype O78 was grown on tryptic soy broth for 24 h at 37°C. Infectious bronchitis virus Arkansas strain 99 (ATCC# VR-841)) was used in this study. The virus was passaged three times in 10-day-old embryonated eggs by allantoic route. The dose of the inoculum used in birds was 10^6 mean EID_{50}/mL.

Experimental design. One hundred and twenty, seventy-two-week-old adult SPF layer chickens were used in this study. The birds were reared in individual layer cages on layer feed under the RAR facility. All the birds were tested negative for ORT by bacterial culture of tracheal swabs and for antibodies to ORT by serum plate agglutination test and ELISA. Birds were randomly divided into groups I through VI containing twenty birds each. Birds in groups I, III, IV, and V were inoculated intra-tracheally with 10^6 mean EID_{50} of IBV. Birds in groups IV and V were aerosolized with one liter each of $2x10^8$ CFU/mL of *E. coli* serotype O78 using a power sprayer. On 5-day post IBV/*E. coli* infection, birds in groups II, III, and V were inoculated intra-tracheally with one mL of sterile phosphate buffer saline (PBS) containing 10^8 CFU of ORT/bird. Birds in group VI were inoculated intra-tracheally with 1 mL of sterile PBS/bird and used as sham inoculated controls.

After the inoculation, the birds were observed daily for clinical signs and mortality was recorded. Two birds from each group were euthanized on days 2, 4, 7, 14, and 28 days post-ORT-infection and necropsy was conducted. Gross lesions were recorded in each bird. Tissues were collected in sterile Whirl-Pak® filter bags for bacteriologic examination or in 10% neutral formaldehyde for immunohistochemistry and histopathology. Samples of trachea, lungs, liver, spleen, kidney, oviduct, and bursa were homogenized and inoculated on SBA and MacConkey agar for isolation and identification of ORT and *E. coli* respectively. In addition, sterile swabs were collected in PBS from air sacs, hock joint, and proximal tibial bone marrow for isolation and identification of ORT and *E. coli*. Samples of trachea and lungs were homogenized in 3 mL of sterile PBS containing penicillin-streptomycin and egg-inoculated for isolation of IBV. Infectious bronchitis virus was later confirmed by RT-PCR. Serum samples were collected

from treatment and control groups at 7, 14, and 21 days post-infection. Sera were examined for the presence of antibodies against ORT by ELISA. The hematoxylin and eosin (H&E) staining technique was used for histopathological examination. A scoring system was used to evaluate histopathological changes in different tissues for analysis and comparison. Trachea, lungs, liver, spleen, kidney, and oviduct were subjected to immunohistochemistry for detecting ORT antigens.

RESULTS

Birds infected with ORT alone showed no clinical signs or mortality. Mortality was also absent in birds of groups infected with IBV and IBV+ORT. In birds infected with IBV alone the clinical signs were mild varying from ruffled feathers and droopiness, whereas the birds infected with IBV+*E. coli* showed more pronounced clinical signs like nasal discharge, droopiness, and off feed. Two birds in group IV and three birds in group V succumbed to death on 2-day-post IBV+*E. coli* infection. Following ORT infection, no mortality was noticed in any groups until 14 day-post infection. One bird each succumbed to death on 14 and 16-day-post-ORT-infection in IBV+ORT and IBV+*E. coli*+ORT infected groups. An acute drop in egg production was noticed in all the IBV infected groups.

On necropsy, no gross pathological changes were noticed in any of the tissues in birds infected with ORT alone. Airsacculitis, lung congestion, and pericarditis were noticed in most of the birds infected with IBV+*E. coli* and IBV+*E. coli* +ORT infected birds on 2 and 4 days-post- infection. Moderate peritonitis was noticed in two birds necropsied from IBV+*E. coli*+ORT infected group on 7 and 14 days-post-ORT infection. One bird from group V, which died on 16 day-post-ORT infection showed pericarditis, perihepatitis, and severe peritonitis. From birds of group II, ORT was isolated from sinus, trachea, air sacs, liver, and kidney. In group IV and V, ORT was isolated from all the above organs as well as from lungs and oviduct. Results of bacterial isolation showed presence of ORT in the infraorbital sinuses even at 28 days post-infection in 20-50% of the ORT infected birds.

On histopathological examination, tracheal tissues of birds in group I to V showed varying degrees of infiltration of lymphocytes, plasma cells, and macrophages in the lamina propria. Lymphoid aggregates were present in severely affected ones which caused the mucosa to bulge into the lumen. A few of the glands were dilated and empty and the cilia were disrupted. Birds in groups IV and V had lymphoid tissue associated with the bronchi (BALT) or lymphoid aggregates in the lung parenchyma. There were moderate numbers of necrotic cells in the BALT. There was focal area of fibrin exudation and infiltration by small number of inflammatory cells in the lungs in birds infected with IBV+ORT and IBV+*E. coli*+ORT. Liver sections showed infiltration of portal tracts by varying number of lymphocytes and macrophages and fibrin deposition. Kidneys showed multiple small interstitial filtrations by lymphocytes and macrophages and mineralized casts in renotubules. Spleen showed varying lesions from moderate lymphoid depletion to severe lymphoid depletion and fibrin deposition. The lesion score was greatest for the groups infected with ORT+IBV+*E. coli*. Immunohistochemical staining revealed the presence of ORT antigens in tissues such as trachea and lungs, liver, spleen, and oviduct of birds. All birds infected with ORT became seropositive to ORT by ELISA on day 7 of infection. The ORT antibody titers remained high even on 28 day post-infection.

DISCUSSION

In this study the authors investigated the pathogenesis of a potential pathogenic isolate of ORT isolated from a severe field outbreak in layer chickens. The birds inoculated with ORT alone or with IBV did not show any clinical signs, gross pathological changes, or mortality, but microscopical lesions were present. But co-infections with IBV and IBV+ *E. coli* exacerbated the clinical signs and lesions varying from tracheitis, airsacculitis, and pericarditis. When co-infected with IBV and *E. coli*, ORT-infected birds did show signs of peritonitis and mortality over a period of time similar to that observed in ORT field outbreaks in layer birds (6).

The results of our isolation, histopathology, and immunohistochemistry studies suggest that the pathogenesis of ORT is less severe in layer chickens when compared to previous reports in turkeys and broiler chickens (4). Experimental infections with ORT in previous studies showed a very low incidence of respiratory lesions in broiler chickens but a co-infection with IBV did produce severe respiratory lesions. Airsacculitis was noticed in experimental infections with IBV alone but no mortality was reported with IBV Arkansas strain in nine-day-old leghorn chickens. However, co-infections with IBV and *E. coli* in the similar experiment reported severe lesions and 10% mortality (1). In a previous study conducted in 26-day-old leghorn chickens, the microscopic lesions due to ORT infections were restricted only to one day post-infection (8). The differences found in the current study and the previously cited studies may be attributed to the age of the birds used or tolerance to ORT by adult layer chickens or in the virulence of the strain of ORT used.

The leghorn chickens were found to be less susceptible to ORT infection than broilers than turkeys (9).

The overall score of histopathological lesions in the present study suggests an enhancement of ORT pathogenesis by a concomitant IBV+*E. coli* infection. In a similar study with five-week-old turkeys experimentally infected with ORT and *E. coli*, *E. coli* was found not to enhance the pathogenicity of ORT. But with concomitant *E. coli* infection in 38-day-old broiler chickens noticed an increased severity of lesions associated with ORT (4).

In the present study, we isolated ORT from air sacs and trachea even after 14 day post-infection and from sinuses even on 28 day post-infection. In a previous study ORT could not be isolated at time points later than two days post-infection from 26-day-old leghorn chickens (8). The bacterial isolation results we obtained indicate that ORT could persist in layer birds for longer periods especially in the sinuses without producing any clinical signs or gross pathological lesions. It could be assumed that ORT would colonize in the nasal sinuses in adult chickens for longer periods and would cause a severe respiratory disease when the predisposing conditions prevail. ORT antigen could be detected in a few occasions in the liver and spleen indicating a possible systemic bacterial infection. This suggests that under field conditions this isolate of ORT may become an invasive agent in adult layer chickens.

In conclusion, the results of our study suggest that ORT+IBV+*E. coli* co-infection is likely to aggravate ORT pathogenesis in adult layer chickens. In layer chickens ORT can persist in tissues for longer periods without causing much severe respiratory pathogenesis and predisposes birds to develop even peritonitis and death. More studies need to be conducted on defining the chronic role of ORT as a pathogenic agent in layer chickens along with other infectious agents.

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SELECTED EGG LAYER CASES

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CASE REPORTS

Many of the intriguing problems seen in layers are due to nutritional in nature either due to feed mill errors, lack of proper absorption of nutrients, or a lack of physical delivery of feed. The lesions seen are often quite common but the stories of how the lesions developed are not.

Copper sulfate toxicity. A flock of 100,000 DEKALB DK layers experienced a mortality increase from the normal of 15 per day to 1000 in one day, two days after a delivery of a fresh batch of feed. The producer submitted 20 fresh dead birds to our laboratory. The birds were all in full production with full crops of feed. The most startling lesion was the finding of aqua blue gizzard contents and edema between the gizzard wall and the lining.

Upon investigating what had happened, it was found that the producer had been visited by the breeder company representative who diagnosed crop mycosis and recommended adding two pounds of copper sulfate per ton. The producer called the feed mill to add this medication to the next load of feed. The person taking the call wrote down "add 2 lbs. copper sulfate per ton" instead of writing out "two pounds per ton". The dispatcher gave the note to the feed mill operator. The feed mill operator read "21" lbs. per ton and the damage was done. A total of about 3% of the layer flock was lost due to the error.

Low calcium intake, unequal feed system delivery. A 60-week-old W98 layer flock began experiencing higher than normal mortality. The dead birds were being found on the feed return side of the chain feed system. Necropsies of fresh dead at the farm showed a dramatically enlarged proventriculus and soft bones. The first impression was that tumors had invaded the proventriculus and resulted in a lack of appropriate proventricular secretion and malabsorption. Histopathology was needed to determine that the proventricular enlargement was due to hypertrophy and hyperplasia of the oxynticopeptic cells and tubular glands.

Upon evaluating the situation, it was found that the running time and speed of the feeder for the feeding system was insufficient to supply the birds on the return side with sufficient feed to supply the birds' needs. Apparently in this case, the birds responded to low calcium intake by attempting to increase proventricular secretion and calcium absorption by building proventricular cell tissue resulting in proventricular hypertrophy.

Stunted chicks. Young egg-type chicks of various strains suffered a dramatic problem with poor weight gains and uniformity beginning at two weeks of age and noticeable well into the pullet grow cycle. No significant lesions were seen during necropsy other than low weights.

Many man hours and expense was done in an effort to determine if feed, IBD, reovirus infection, other viral infections, *Salmonella* infections, vaccination reactions, etc., took place over a period of several months. None of the infectious disease investigations showed any abnormalities. Feed assays for protein, sodium, calcium, phosphorus, animal protein quality, soymeal quality, and rancidity failed to show any problems. Amino acid assays were not routinely done at the time of this problem as they are today.

Eventually, an examination of the methionine addition system showed that the system supplying one of the two mixers was completely plugged and not delivering any added methionine resulting in half the normal amount.

Acute increase in mortality due to cannibalism. A five-house layer complex with various strains and ages of layers began experiencing an alarming increase in mortality due to cannibalism in all five houses within one week's time. An investigation of the layers showed normal feeding, lighting, watering, and ventilation management. An increase in activity was seen however with a slight increase in feed consumption, lowered egg production, and lowered egg weights.

Necropsies of fresh dead showed lesions of cannibalism but no evidence of enteritis or parasitism. Feed assays showed normal results for protein, calcium, sodium, and phosphorus.

A meeting with the feed company revealed that they had switched to a new supplier of animal protein two weeks prior to the problem starting. Analysis of this source of protein showed normal protein content but the protein digestibility was found to be much lower than 90% (normal is above 90%) while the previously used source had a protein digestibility of 98%.

Layer malabsorption syndrome. Eggs from a 38-week-old W98 layer flock were found to have very pale yolks after hard-cooking. Some had a pale center with normal yolk color surrounding; some had normal colored centers and pale outer layers; while some had a ring of pale yolk in between normal colored yolk. Flock production and egg weights had dropped significantly as well. Necropsies revealed very thin intestines containing orange mucoid material. Ova were very pale. The flock recovered fully in about three to four weeks.

Histologic findings included stunting of villi in the intestine plus lymphocytic enteritis. Electron microscopy studies showed a small virus present. Studies on this possible enteric agent are continuing.

EVALUATION OF A MULTI-SPECIES AVIAN INFLUENZA ANTIBODY ELISA

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ABSTRACT

IDEXX Laboratories has developed a multi-species avian influenza antibody ELISA test kit, and has evaluated performance characteristics, including specificity, reproducibility, and analytical sensitivity in many avian species. Species tested to date include chickens, turkeys, ducks, geese, and ostriches. The test has demonstrated detection of antibodies to at least 14 AI subtypes and has demonstrated 100% specificity in chicken, turkey, and ostrich populations tested to date. Because it can provide excellent sensitivity and specificity in combination with ease of use and high throughput testing, the IDEXX Multi-species ELISA can become an important diagnostic tool for monitoring exposure to avian influenza in poultry flocks and wild populations.

INTRODUCTION

Avian influenza (AI) virus is known to infect domestic poultry as well as wild avian species. The disease is characterized by a wide range of responses which include virtually no clinical signs to high mortality. Avian influenza subtypes, therefore, are classified as either highly pathogenic (HPAI) or of low pathogenicity (LPAI) (1). There are multiple hemagglutinin (HA) and neuraminidase (NA) subtypes that represent much genetic diversity in the virus resulting in broad multi-species transmissibility. The 15 viral subtypes of hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have all been found in wild waterfowl (2). Detection of AI in wild birds is important because they represent a major reservoir for virus propagation and transmission (2). In addition, the virus poses a significant threat to the poultry industry and can cause serious economic impact.

Several serological methods are currently used to determine influenza infection in birds: agar gel precipitation (AGP), hemagglutination inhibition (HI) which is complex due to the many viral HA subtypes, and enzyme-linked immunosorbent assay (ELISA). Detection of antibodies from multiple species using ELISA has been limited due to lack of homology between avian immunoglobulin sequences. However, viral surveillance of other avian species is important to prevent viral crossover and major outbreaks of HPAI strains.

IDEXX has recently developed a new avian influenza antibody ELISA test (AI MultiS-Screen) that can detect exposure to avian influenza subtypes across multiple avian species. The ELISA test successfully detects antibodies to avian influenza in serum from chickens, ducks, and turkeys. To assess performance of the multi-species ELISA, the sensitivity and specificity was determined for duck, chicken, and turkey populations. Temporal sensitivity was analyzed for chickens. In comparison with the performance of current commercially available diagnostic tests, this multi-species ELISA performs as well as or better than other single species tests. These studies provide an important diagnostic tool for monitoring exposure of poultry flocks to all influenza subtypes as well as monitoring wild avian species infections.

MATERIALS AND METHODS

Data for this study was collected from chicken, turkey, duck and ostrich serum samples obtained from the United States, Europe and Asia. Each sample was tested on the new IDEXX AI MultiS-Screen ELISA. Avian serum samples were diluted 1:10 in sample diluent and added to the ELISA plate at 100µL/well.

Following a 60 minute incubation at room temperature, plates were washed five times with PBS-Tween. Detection antibody (100uL) was added to each well and incubated at room temperature for 30 minutes. Plates were then washed and developed with 100µL/well of TMB substrate for 15 min followed by addition of 100µL/well of stop solution. An absorbance reading at 650nm for each sample was obtained.

Interpretation of results. The presence or absence of antibody to AI was determined by the sample OD/negative control OD (S/N) ratio for each sample. S/N ratio = (sample mean A650)/(negative control mean A650). Samples with an S/N ratio ≥ 0.50 are considered negative. Samples with S/N values < 0.50 are considered AI antibody positive.

Chicken samples. To determine the day of seroconversion following exposure to AI subtypes, four specific-pathogen-free chickens per group were inoculated i.m. with one of four influenza subtypes; H1N1, H3N8, H5N2, and H7N3. Chickens were bled via wing web vein to obtain serum before inoculation (day 0) and at days 5,8,13, 21, and 28 days post inoculation. Sera was collected and pooled from two birds on each bleed date. To date, 1838 negative chicken serum samples have been run on the ELISA to test specificity. Serum from chickens infected with 14 different AI subtypes were obtained from NVSL (Ames, IA). Each of the chicken serum samples was also tested on a commercially available AI ELISA kit, following the protocol outlined in the package insert.

Turkey samples. To test specificity, 757 negative turkey samples were analyzed, and 450 positive turkey samples were tested for sensitivity.

Duck samples. Negative duck samples (n = 237) and 60 positive duck samples have been evaluated.

Ostrich samples. Specificity testing was conducted on 368 negative ostrich samples.

RESULTS

Sensitivity of the AI MultiS-Screen ELISA for chicken populations was evaluated with experimental exposure temporal samples. Seroconversion was seen as early as eight days post inoculation, with AI subtype H7N3 showing the earliest immune response. H3N8 (a non-avian isolate) displayed a seroconversion date of 21 days post inoculation. The specificity of the ELISA screen for chicken samples tested to date (n=1838), was 99.7%.

Specificity and sensitivity were also assessed for turkey serum samples. The AI MultiS-Screen ELISA had a sensitivity of 98% on an AI vaccinated turkey population (n = 450) and specificity of 100% on a negative turkey population (n = 757).

Duck serum populations tested resulted in a specificity value of 100% (n = 237) and sensitivity for an AI vaccinate duck population of 91.7% (n = 60). The five discrepant vaccinate duck samples that tested negative on the AI MultiS-Screen ELISA also tested negative by HI.

Testing with the AI MultiS-Screen ELISA on ostrich sera showed 100% specificity (n = 116).

The AI correlates well with HI and AGP testing methods (100% and 99% correlation to the AI MultiS-Screen ELISA respectively).

DISCUSSION

The results discussed here illustrate sensitivity and specificity values determined on populations to date for chicken, turkey, duck, and ostrich avian species. The AI MultiS-Screen ELISA was able to detect AI seroconversion in chicken as early as eight days post infection and had a specificity for chicken serum of 99.7%. Furthermore, the test displayed broad subtype detection and is capable of detecting chicken serum antibodies to at least 14 different viral subtypes tested.

Analysis of turkey serum showed that the AI MultiS-Screen ELISA detected AI antibodies with a sensitivity of 98.0% and specificity of 100% for the samples tested to date. The assay also had 100% specificity for duck and ostrich populations.

Upon comparison of the AI MultiS-Screen ELISA with other AI antibody testing methods, the multi-species test presented here correlates well with AGP, HI, and a commercially available ELISA, showing 100%, 99%, and 98.6% correlation, respectively among the currently tested populations. These data demonstrate that the AI MultiS-Screen ELISA can accurately and successfully detect AI exposure in several avian species (chicken, turkey, and duck).

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CHICKEN AND INTRA-CLASS INTER-SPECIES TRANSMISSION OF LOW PATHOGENIC AVIAN INFLUENZA: A LABORATORY EXPERIMENT USING BIRDS SOLD IN LIVE BIRD MARKETS

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SUMMARY

Many theories of the modes of transmission of avian influenza (AI) have been presented, but few have been quantified within a flock or live bird market (LBM) setting (1-3). Laboratory experiments are usually done using SPF chickens which are more resistant to disease infection than the breeds sold commercially (4, 5). This study simulated the transmission of AI in an LBM by contact exposure of susceptible birds to inoculated chickens placed in stacked cages. The experiment will provide estimates for the rates of spread within an LBM due to poultry handling practices, direct exposure to feces (or fecal shedding), and aerosolization of the virus within a small space. Chickens, ducks, and quail that were similar in age and breed type of birds sold in LBMs were placed in stacked cages. Two trials were conducted, once using trays between the cages and repeated without using trays between the cages. In each trial, five chickens in one cage were inoculated with H6N2 AI virus on day one. Oral/pharyngeal and cloacal samples were taken daily for 16 days and serum was collected from each of approximately 80 birds in three rooms every seven days until the trial ended at day 16.

Patterns observed from sample data showed that direct fecal exposure was a more efficient mode of AI transmission than handling poultry and aerosol. None of the infected birds presented with any clinical signs. The transmission estimates obtained from this study will be used in a spatial simulation model to evaluate the spread of low pathogenic AI within mixed flocks of poultry in an LBM setting.

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THE POTENTIAL WEAPONIZATION OF AVIAN INFLUENZA VIRUS – A POSSIBLE THREAT TO THE COMMERCIAL POULTRY INDUSTRY

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ABSTRACT

Avian influenza has been responsible for over 250 human cases, including 153 deaths in ten different nations from 2003 till November of 2006. In light of other problems, such as the anthrax attacks which occurred in 2001 in the United States and the search for weapons of mass destruction in Iraq, serious questions have been raised by the government and the public as to whether U.S. adversaries could exploit the influenza virus in such a way that it could be used as a weapon against animals and or people. History indicates that a number of nations did have biological weapons programs that included influenza or even in some cases, avian influenza research. Many of these programs were shut down with the fall of the Soviet Union. With its demise also came the possible dispersion of biological agents and equipment, but more importantly the knowledge base and the people who were responsible for the Soviet research and weaponization programs. Although, the exact nature of current programs are not fully known and cannot be discussed in non-classified environments, biological weapons programs continue today in several threat nations. The questions then remain, can the avian influenza virus be utilized as a biological weapon against the United States or her allies, and if so, what are the threats and the nature of the elements that could indicate that plans are being made to target the U.S.?

INTRODUCTION

Avian influenza is a term that has been confused in much of its meaning due to a lack of understanding by the world press. The disease "avian influenza" can broadly be classified as occurring in two forms, including most commonly that which occurs regularly and solely in avian species. Since 1997 a second form of the disease has emerged, which resulted when the virus achieved limited success in crossing into humans. Most recently, from the period of 2003-present, the H5N1 strain of the virus has caused a total of 258 recognized cases, including 153 deaths and an unknown number of unrecognized cases (7). With the emergence of avian influenza and possible pandemics as public issues, and in reaction to the post -9-11 anthrax attacks in the United States, the intelligence community and various governmental agencies began to re-review documents recovered from the former Soviet Union and subsequently review al-Qaeda documents seized in Afghanistan and Iraq to determine whether avian influenza is a legitimate threat to agribusiness, the citizens of the United States or her allies.

Some of the findings of this review can be discussed in non-classified environments, others cannot. Current intelligence indicates that at least twelve nations currently maintain probable biological weapons research programs (3). Most of the programs are small and restricted to laboratory research. Other programs are much larger and pose a more direct threat to the U.S. Of those nations having programs, nine including Algeria, China, Cuba, Egypt, Iran, North Korea, Russia, Sudan and Syria are considered potential adversaries. The exact status of another program (Libya) is not confirmed and three programs (India, Israel, and Taiwan) are being conducted in allied nations. Besides these programs, non-state based adversaries, including al-Qaeda and possibly Hezbollah, have had biological weapons contacts with U.S. adversary states or scientists from the former Soviet or South African biological weapons programs. Documents seized in Afghanistan in 2001 and currently contained within the Department of Defense's Harmony Database indicate that multiple pathogens, including avian influenza were being discussed by al Qaeda as possible weapons against the United States. Although, there is direct evidence of chemical weapons testing by the group, there is no openly available direct evidence that any biological weapons development ever occurred. Of the nations mentioned previously, Russia's current status is perhaps least clear, since a substantial avian influenza weapons program was documented in the USSR and materiel and scientists that were associated with the program remain in part unaccounted for in former Soviet Block nations, including Russia, Kazakhstan, and Uzbekistan (3). The

status of other materials, including avian influenza virus previously documented in Iraq remain unknown.

LOGISTICAL CONSTRAINTS TO BIOLOGICAL WEAPONS PROGRAMS

How does one know a biological weapons program when it is seen? What are its telltale characteristics? Biological Weapons programs (BWP) can take many forms, including those aimed solely at people, plants, infrastructure or animals or those dual purpose programs utilizing zoonotic pathogens, which have the potential of causing disease in both humans and animals. State based programs, such as the most massive example, which was contained within the multi-target (people, animal, infrastructure) multi-agent Soviet Biopreparat program, tend to be characterized by large bureaucracies, huge expenditures for people and equipment, massive amounts of infrastructure and extensive and aggressive procurement programs designed to gather new and novel highly pathogenic organisms (1). Since the fall of the Soviet Union, this paradigm has shifted dramatically, in that BWP are by necessity smaller and therefore less visible to the U.S. Intelligence Community (IC). The main reasons for maintaining a program of reduced scale are economics and survival. Less visible programs are less likely to be discovered and destroyed by the U.S. Reviews in post invasion Iraq indicate that the formerly active and relatively large BWP (Pre-Gulf War) changed dramatically in the post war period, when Saddam Hussein widely dispersed potential agents and equipment in response to the presence United Nations (UN) Inspectors and out of fear of attacks by the U.S. Rather than maintaining an active or even a suspended program, the strategy for Post-Gulf War Iraqi BWP was to maintain a "potential" or inactive but easily reactivated system, where biological weapons agents were stored in legitimately operating laboratories, such as veterinary diagnostic or medical research laboratories. Interestingly, prior to the Iraq war, UN inspectors did uncover at least one facility where avian influenza research was conducted. The exact nature of the research was never determined, but as is so often the case, the research could have easily been adapted to offensive applications. In many nations, so called dual use technology has become the rule, rather than the exception. The dilemma of not understanding how to accurately predict real intentions continually causes both the scientific and IC communities to struggle with determining where legitimate research ends and biological weapons programs begin.

Besides delivery methods and systems, which remain a huge obstacle for any adversary to overcome, significant frictions also remain in the scope and nature of the actual biological research needed to locate and isolate the ideal weapons grade pathogen. Although the scale of production can remain small, an inescapable constraint to any program is that a sufficient volume of biological agent must be obtained for there to be any reasonable expectation, that if delivered, a sizeable negative effect will be produced within the midst of an enemy. The scenario familiar in the movies where one infected person or animal begins a plaque that sweeps a nation is most cases truly a work of fiction. For an adversary to successfully destroy or even cause a significant negative effect there must be 1) the proper agent, 2) in sufficient volumes, and 3) delivered over a wide enough geographical region to make early detection, response and containment difficult or impossible. Anything less may cause momentary panic or negative economic impact, but it will not result in irreparable destruction. By their very nature, these constraints leave tell-tale evidence, which if successfully gathered and interpreted properly, can tell much about the potential intentions of an adversary.

Avian influenza virus, like other viruses has several constraints which must be overcome before it could be used as a biological weapon. Krug (5) described how a lethal influenza virus could be generated using a reverse genetic system. The genetic structure of the 1918 strain has also been presented by Taubenberger, *et al*., (6) and the full genome is on file in the GenBank database. In addition, genomic information is available for the H7N7 strain of influenza which has shown some ability to be transmitted from birds to humans (2). Concern has been voiced in a number of publications and editorials that terrorists could use this information to reconstruct the 1918 influenza virus (4). While possible, using current methods of construction, this task would prove extremely difficult, although not impossible to execute. Other constraints include virus isolation, cultivation and purification, stabilization and preparation so that the correct particle size could be produced for aerosolization. Once, overcome an adversary would then have to deal with the issues off efficient delivery without detection. Collectively these tasks make it difficult for any adversary, other than those who are state-based or very well organized and financed, to deliver an effective blow to the United States sufficient in size to cause widespread damage.

CURRENT POTENTIAL THREATS

The Department of Homeland Security (2006) produced a Red Cell Report which discussed three potential scenarios for weaponizing the avian influenza virus. Although, marked "Unclassified", the report is designated "For Official Use Only" and therefore its contents cannot be discussed fully. Some of the summary conclusions indicated that the primary

motivation for using the virus would be to inflict economic damage, since alone or unmodified the pathogen was unlikely to threaten the long term public health or irreparably damage the food supply. Of the three scenarios discussed in the document, the most likely was considered diversion of infected materials from endemic areas, such as occur in Asia, Iraq or parts of Africa. Once collected, the material would have to be successfully transported into the U.S. and successfully delivered in an appropriate manner to poultry producing regions so as to cause a sufficiently widespread infection. As previously mentioned, although not impossible, fortuitously for the sake of the nation, significant constraints remain which lower the probability of adversarial success.

CONCLUSIONS

Although, there are no current known threats where avian influenza virus is implicated, the potential, though remote does remain. Continued vigilance is essential for the detection of potential biological attacks, including those involving the use avian influenza virus. The IC must work more closely with the veterinary and public health communities, as well as agribusiness. Any avian influenza outbreak, regardless of its source has the potential of causing catastrophic losses to the U.S. poultry flock and devastating losses locally, regionally or nationally. The continued availability of a safe, economical and dependable food supply, of which poultry products are an important ingredient, is probably not threatened at this time. Its actual continuation in the future is however highly dependent on correct assessments as to new and emerging threats and the detection of shifting potential vulnerabilities. A system capable of early detection and containment, containing sufficient numbers of trained professionals is imperative. Once created on a national basis, it will have capabilities at the local level for dealing with any animal emergencies, including those resulting from naturally occurring diseases or those borne from those who do evil.

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TRAINING RESOURCES FOR PREVENTION AND INTERVENTION STRATEGIES DURING AVIAN INFLUENZA

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OVERVIEW

Avian influenza (AI) has emerged as one of the most serious animal health emergencies in recent memory, and it threatens to be one of the most serious public health threats in the 21st century. Because of the global impact of the virus, there has been a rapid dissemination of information, especially via the internet. However, much of this information is either inaccurate or biased for a particular purpose. Numerous governmental agencies are involved in the dissemination of factual educational information, including the USDA, CDC, DHS, FDA, and international organizations such as the WHO, FAO, and OIE, which is the World Organization for Animal

Health that monitors animal diseases worldwide and sets international standards and policies for animal trade and products (www.oie.int). Accurate educational information is best achieved when governmental agencies with oversight responsibilities partner with scientific institutes and universities that can lend expertise in educational dissemination. The Center for Food Security and Public Health (CFSPH) (www.cfsph.iastate.edu), College of Veterinary Medicine at Iowa State University, is an example of a successful university-based Center that collaborates with a number of national and international governmental agencies, and universities and departments to provide educational resources for disease prevention and detection, including avian influenza. The Center for Food Security and Public Health (CFSPH) is a CDC Specialty Center in Public Health Preparedness for Veterinary Medicine and Zoonotic Diseases with a mission to increase national preparedness for accidental or intentional introduction of disease agents that threaten food production or public health.

The Institute for International Cooperation in Animal Biologics (IICAB**),** Iowa State University (http://www.cfsph.iastate.edu/IICAB/default.htm) is a division of the CFSPH. IICAB is an OIE Collaborating Center for Diagnosis of Animal Diseases and Vaccine Evaluation in the Americas; a designation shared with the USDA laboratories in Ames, Iowa. IICAB has the goal of improving animal health worldwide through the use of effective vaccines and diagnostics, and provides training, access, and expertise via scientific training for government and industry personnel from the U.S. and other countries, by hosting scientific meetings, and by providing scientific expertise to the member countries of the OIE. An example of a long-standing educational partnership is the OIE Veterinary Biologics Training Program administered by CFSPH and IICAB which is a course co-sponsored by the USDA Animal and Plant Health Inspection Services (APHIS) Center for Veterinary Biologics (CVB) and National Veterinary Services Laboratories (NVSL), and the Iowa State University (ISU) College of Veterinary Medicine. The training includes lectures and laboratory workshops in immunology with an emphasis on resistance to infectious disease; procedures for ensuring vaccine safety and efficacy; reviews for licensing/registration and testing of veterinary biological products and the compliance process; and laboratory testing of biologics and diagnostic test kits.

The CFSPH website contains a feature on avian influenza (http://www.cfsph.iastate.edu/Feature/aiIn Depth.htm) that contains basic information, technical fact sheets, key points, comparison information with Newcastle disease, biosecurity and disinfection information, and avian influenza information for scientific and medical/veterinary audiences, as well as the general public. Resources also include identification and testing, personal protective equipment use, and a resource for control strategies, including vaccination and/or eradication, during an avian influenza outbreak. The website also contains disease lesion images of avian influenza and other diseases that are diagnostic differentials in poultry (http://www.cfsph.iastate.edu/DiseaseInfo/ImageDB/ imagesAI.htm). Image acquisition is funded by a USDA Higher Education Challenge Grant in partnership with the ISU Department of Veterinary Pathology, USDA, Plum Island Animal Disease Center (PIADC), Armed Forces Institute of Pathology (AFIP), and numerous contributors.

The CFSPH/IICAB conducted a series of Avian Influenza – Newcastle Disease Virus Diagnostic Workshops in cooperation with the USDA Animal and Plant Health Inspection Service, National Veterinary Services Laboratories (USDA-APHIS-NVSL), (NVSL), and the USDA Agriculture Research Service's Southeast Poultry Research Laboratory (USDA-ARS-SEPRL), and the attendees included government officials from 60 countries. USDA is funding the conversion of these workshops into DVDs that will be further disseminated to officials, scientists, and technicians in various countries. The DVDs will include lectures on avian influenza including clinical aspects, differential diagnosis, diagnostic tests, molecular diagnostics, and virus characterization; specifically viral isolation, antigen detection, agar gel immunodiffusion, hemagglutination inhibition, ELISA, AGID, and molecular diagnostics, including real-time RT-PCR (RRT-PCR), and neuraminidase inhibition.

Recently, lectures, interviews, and reference materials on important issues related to vaccination decisions world-wide for control and eradication of avian influenza are being assembled in a DVD format to provide facts and expert opinions regarding value, risk, and impact for AI vaccine decisions. This project is being conducted by the CFSPH/IICAB and sponsored by USDA with cooperation of the OIE. The reference material will be available for four target audiences of policy decision makers and field operations personnel including: 1) country leadership in government, industry, and agencies; 2) leadership in government agencies (agriculture, finance, public health, environment, transportation, etc); 3) ministries of agriculture and health; and 4) epidemiologists, veterinarians, field staff, and laboratory personnel. The project will: 1) provide an international and national expert panel assembled in January 2007 (International Poultry Expo, Atlanta, GA) and March 2007 (OIE/FAO/IZSVe AI meeting, Verona, Italy) to give scientific lectures and a round table discussion videotaped for a DVD format; 2) produce presentations

for the four audience groups listed above; 3) develop case studies; 4) provide a reference guide for AI vaccine decisions; and 5) document current and developing technology. Topics will include: infrastructure of official services required for vaccination; 2) diagnostic infrastructure, surveillance, disease reporting; 3) structure of the poultry industry and husbandry; 4) role of the poultry industry in vaccination decisions and implementation; 5) epidemiological factors influencing vaccine decisions; 6) goals of vaccination; and 7) vaccination procedures and monitoring programs. This will be a comprehensive resource that will be disseminated by the USDA and OIE.

The last example is a CFSPH web-based training module on avian influenza and exotic Newcastle disease for a course entitled "Foreign Animal Disease Awareness." Information on Avian Influenza also can be found in a similar web-based course entitled "Program Diseases: The National Poultry Improvement Plan." Both of these courses were funded by the USDA- APHIS and are required training for all new APHIS veterinarians and animal health technicians. The CFSPH received permission from the USDA to make these courses available as free continuing education for veterinarians on the CFSPH website as the Foreign Animal Disease Awareness Course and Program Disease Course (http://www.cfsph.iastate.edu/CE/courses.htm).

Effective partnerships require significant resources for time, money, and personnel, but the need to provide accurate information is critical, especially in the area of infectious, emerging, and re-emerging diseases. The biosecurity of the U.S. food supply and human health can be assisted by bringing together educators, scientists, and government agencies and officials for educational resources and training to increase awareness, provide accessibility, and train key personnel in disease diagnostics and prevention.

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DIFFERENTIAL INNATE CYTOKINE RESPONSES CORRELATE WITH PATHOGENICITY IN CHICKENS AND DUCKS FOLLOWING INFECTION WITH ASIAN H5N1 VIRUSES

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SUMMARY

The immune system can be divided into two functional components, the innate and adaptive, that differ in their mechanism of pathogen recognition and response. The innate immune response is responsible for detecting invading microorganisms during the initial stages of infection, which is a crucial determinant of disease resistance or susceptibility. Because chickens normally succumb to disease within three to four days after infection with highly pathogenic Asian H5N1 avian influenza (AI), the adaptive immune response likely contributes little to defense against disease. On the other hand, waterfowl, including ducks, are considered natural reservoirs for AI and rarely display clinical signs of disease. The reasons for the differences in susceptibility and pathogenicity of different avian species to different pathotypes of AI are unclear and ill defined. These studies were designed to examine the role of the innate immune response in protection from disease by measuring cytokine expression with RRT-PCR immediately following infection. The results indicate differential cytokine expression *in vivo* between chickens and ducks following exposure to AI. Ducks resistant to disease generally displayed increased cytokine expression, while chickens susceptible to disease tended to exhibit suppressed cytokine expression.

CO-EXISTENCE OF NEWCASTLE DISEASE VIRUS AND AVIAN INFLUENZA VIRUS IN DOMESTIC POULTRY – A PROMOTER TO TRIGGER GENOME RECOMBINATION AND REASSORTMENT EVENT?

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ABSTRACT

Thirty cloacal swab samples were collected from a local chicken farm during the period of November to December 2005. RT-PCR test was performed for avian influenza virus (AIV) and Newcastle disease virus (NDV). Seven (23.3%) and sixteen (53.3%) specimens were positive to AIV and NDV respectively, and one individual was tested to be co-infected with both viruses. Sequence analysis of NDV hemagglutinin-neuraminidase (HN) and fusion protein (F) gene reveals that the isolates showed high homology to those of other highly pathogenic (viscerotropic and neurotropic velogenic) NDVs. The analysis on AIV hemagglutinin gene shows that all AIV position isolates belong to H9 low pathogenic lineage. Neither recombination nor sequence variations were observed in the co-infected sample when compared to the other solely infected samples. In order to assess whether NDV contributes to the increase of likelihood of co-infection and hence re-assortment of AIV genomes, or vice versa within a common host cell, an *in vitro* infection experiment was performed by inoculating both viruses into MDCK cell line. The cells were infected with two MOI $TCID_{50}$ of velogenic NDV and/or H6 and H9 lineage AIV. At 48 hours p.i. 50 single virus plaques were isolated from each set of infection and followed by RT-PCR and sequence analysis.

(The full-length article will be submitted for publication in *Poultry Science*.)

LOCALIZATION OF MONGOLIAN STRAIN H5N1 HPAI VIRAL ANTIGEN IN FORMALIN FIXED AVIAN TISSUE SPECIMENS UTILIZING IUMMUNOHISTOCHEMISTRY (IHC)

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ABSTRACT

Successful localization of H5N1 highly pathogenic avian influenza (HPAI), viral antigen in formalin fixed avian tissue specimens from various viral strains is a highly desirable diagnostic pathology screening tool to preferably be used by pathology investigators at the USDA, APHIS,VS, National Center for Animal Health,(NCAH), Pathobiology Laboratory (PL), General Pathology Pathology Investigations Section (GPPI), in Ames, IA especially in light of the probable increased diagnostic avian pathology requests anticipated by the highly publicized predicted worldwide and national HPAI pandemic.

Previous successful HPAI viral antigen localization in formalin fixed avian tissue specimens collected from 15 week old chickens inoculated with the Hong Kong strain of H5N1 HPAI utilizing IHC was achieved by A. J. Wilson of USDA, APHIS, NCAH, PL, GPPI in 1998 and the results of that study were presented at the 1998 Scientific Meeting of the AAVLD in Minneapolis, MN.

The Mongolian strain of H5N1 HPAI obtained by researchers at NCAH was inoculated into eight 15-week-old chickens in October 2005 and necropsied by Wilson who found numerous gross pathology lesions in all eight chickens necropsied. The respiratory, lymphoreticular, circulatory, and digestive systems were the most significantly affected based on gross necropsy results. Cyanosis of the comb and wattles, multifocal subcutaneous hemorrhage in the musculoskeletal system, severe peritracheal hemorrhage, multifocal pulmonary hemorrhage with edema, marked splenomegaly and multifocal mucosal congestion and hemorrhage in the digestive tract extending from the proventriculus, ventriculus, small and large intestine were observed in all of the eight chickens necropsied in October 2005 at NCAH.

An avidin biotin IHC procedure adapted from a method described by L. Vincent *et al*. in the *Journal of Vet Diag. Invest*. vol 9:191-195, 1997 was performed on the tissue specimens collected in October 2005.

Successful viral antigen localization from the Mongolian strain of HPAI H5N1 was achieved in the respiratory, lymphoreticular, digestive, nervous, cardiovascular, and integumentary system from all eight birds inoculated and later necropsied by Wilson.

The results of this successful initial IHC viral antigen localization screening study conducted by A. J. Wilson and colleagues at USDA,APHIS,VS,NCAH, in collaboration with J. Richt of ARS, and B. Janke and D. Cavanaugh of Iowa State University has successfully shown that IHC can serve as a very valuable diagnostic pathology viral antigen localization screening tool to assist USDA,APHIS,VS,NCAH in meeting the anticipated increased diagnostic workload anticipated in the possible fall 2006 HPAI pandemic threatening U.S. agricultural poultry operations.

NON-PURULENT ENCEPHALITIS WITH MORTALITY AND FEATHER LESIONS IN CALL DUCKS (*ANAS PLATYRHYNCHA* VAR. DOMESTICA) INOCULATED INTRAVENOUSLY WITH JAPANESE H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS

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SUMMARY

One-day-old, two-week-old, and four-week-old call ducks (*Anas platyrhyncha* var. domestica) inoculated intravenously with the Japanese H5N1 highly pathogenic avian influenza virus A/chicken/Yamaguchi/7/2004 isolate (Ck/Yama/7/04) were examined clinically, pathologically, and virologically. Clinically, the birds exhibited mild to severe neurological signs and corneal opacity. All birds in the one-day-old group and one bird in the four-week-old group, died within four days after the virus inoculation. Histological changes were characterized by severe non-purulent encephalitis and necrotic lesions of the feather epithelium on day three post inoculation or later. Focal necrosis of the heart, pancreas, skeletal muscle, and corneal epithelium were also observed. Viral antigens were detected in association with necrotic changes. Viruses were isolated from all examined organs including the skin with many feathers. These results suggest that Ck/Yama/7/04 has a pathogenicity that causes mortality, non-purulent encephalitis, and feather lesions for call ducks. Feather lesions with viral antigens and the virus isolation from the skin suggest that Ck/Yama/7/04 has a predilection for feathers in call ducks. This raises the possibility that the viruses may be released from feathers, leading to possible viral transmission to other birds or mammals.

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

RETROSPECTIVE REVIEW OF AVIAN INFLUENZA H5N1 CONTROL IN HONG KONG 1997-2007

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ABSTRACT

The 1997 highly pathogenic avian influenza (HPAI) H5N1outbreaks in Hong Kong (HK) caused high mortality in chicken farms and 18 human cases with six deaths, which was the first report of lethal HPAI H5N1 infection in humans. Although HK subsequently experienced further HPAI H5N1 outbreaks in 1997, 2001, 2002, and 2003, there has been no outbreak in commercial poultry farms for four years since February 2003. In these years, substantial and comprehensive control and surveillance systems have been progressively strengthened and enforced with the use of vaccination with Nobilis IA inac® (H5N2 inactivated oil emulsion vaccine) and Nobilis IA+ND inac® (H5N2 and ND inactivated oil emulsion vaccine) in commercial chickens as an additional measure to control H5N1 HPAI in HK. In particular, emergency vaccination used in the face of outbreaks on unvaccinated farms, coupled with selective culling and

stringent biosecurity, resulted in elimination of H5N1 virus infection from these farms in 2003.

In the face of the increased human cases and potential pandemic threat of HPAI in Asia, this paper reviews and describes the major control and preventive measures in these 10 years against incursion of HPAI that have been or will be introduced in HK.

INTRODUCTION

Since the mid-1970s, Hong Kong (HK) has long conducted studies on influenza ecology and functioned as an influenza sentinel post for southern China region (13, 14). In 1997, 2001, 2002, and 2003, HK experienced highly pathogenic avian influenza (HPAI) H5N1 outbreaks, among which the 1997 outbreak caused 18 clinical human influenza cases with six deaths and historically marked the first lethal avian influenza (AI) H5N1 infection in humans (8, 9). Due to the zoonotic concern in 1997, all poultry in the markets and farms were slaughtered to stop further exposure of human (8). The H5N1/97 virus was thought to be a triple avian virus re-assortment of which the hemagglutinin gene was derived from A/Goose/Guangdong/1/96, a H5N1 virus from geese, the genes encoding the internal proteins were from an A/Quail/HK/G1/97-like H9N2 or A/Teal/HK/W312/97-like H6N1 virus and the N1 gene was from a Quail H6N1 origin (6, 16).

In 2001, multiple genotypes of H5N1 viruses were isolated in the poultry retail markets in HK and chickens soon died market by market despite no outbreak found in commercial poultry farms. Nevertheless, all poultry in the markets were slaughtered to prevent the spread of the disease and possible further re-assortment for unwanted strains (14).

In between February and May 2002, a new subset of genotypes of H5N1 viruses was found in the retail markets again and the control measures was again by culling of poultry in affected markets. Despite the initial stamping out policy, the repeated incidents of H5N1 outbreaks might suggest a potential HPAI problem would be continuing. Surely enough, H5N1 viruses were found in waterfowl in two recreation parks in late 2002 and a few chicken farms in early 2003 in Hong Kong.

In April 2002, an AI vaccination trial with Nobilis IA inac (H5N2 inactivated oil emulsion vaccine) was initiated in the chicken farms in Paksha (a geographically high risk area for HPAI in HK). This program had progressively become a universal vaccination policy for the local chicken industry in June 2003.

The local poultry industry continues to enjoy free of HPAI H5N1 status with the same vaccination program in place despite various Asian AI outbreaks in the recent time (9). A retrospective review of the H5N1 HPAI experience in HK is discussed in a field perspective in this paper.

Monitoring and surveillance in Hong Kong. Local poultry are monitored throughout their entire production life. Samples are collected from every single batch of pre-sale local poultry to check for exposure to H5 avian influenza virus in sentinel chickens or for H5 antibody levels in post-vaccinated chickens. Prior to sale, 60 samples per batch of commercial chickens have cloacal swabs collected and tested for H5 genome in addition to the serological test in case the sentinel turns out to be positive. Routine virological monitoring and surveillance is done in the live poultry markets, local farms, pet bird market, recreation park, wild birds, other bird collections and dead bird referral (8).

Sentinel. Sixty (60) sentinel birds are placed in each flock randomly with marks for differentiation from vaccinated birds. The sentinels will be vaccinated with vaccines against major endemic infectious diseases except AI vaccines. Placing sentinels is a sensitive tool for early detection of AI incursion under field conditions and differentiating infected from vaccinated birds.

Vaccine. The birds on all commercial chicken farms are vaccinated with either or both Nobilis IA inac (H5N2 inactivated oil emulsion vaccine) and Nobilis IA+ND inac (H5N2 and ND inactivated oil emulsion vaccine) produced by Intervet. The vaccine is recommended to keep at 2-7 ^{o}C for storage and pre-warm to room temperature before use. The vaccine is administered subcutaneously or intramuscularly. Two vaccinations are given with four weeks interval to local native broilers which are marketed at 60-100 days of age (9). A third dose is used if chickens failed to show satisfactory antibody response after the second dose, which is usually caused by poor vaccination techniques or biological variations among individual chickens.

Serology testing. Standard hemagglutination inhibition (HI) test (1) is used to check for H5 antibodies, especially in sentinel birds. The HI test is also a tool to monitor the vaccination techniques in vaccinated birds under field conditions (8, 9). As the neuraminidase antigens (N2) in the vaccine differ from that of H5N1 (N1), 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 (2). However, N1 or N2 testing have never been done

as routine monitoring for the purpose of DIVA. Sentinel birds are the DIVA in HK.

H5 genome detection. Cloacal swabs were collected for rapid diagnosis for viral genomes using real-time RT-PCR (15) before being subjected to virus isolation for final confirmation. Directigen® is used at discretion and NASBA (3) was used before real-time RT-PCR was introduced.

Virus isolation. Virus isolation (1) is used for final confirmation and as part of the surveillance as described above. It also plays an important role in conducting genetic and antigenic characterization of viral isolates to study the evolution of avian influenza virus.

CLINICAL AND SURVEILLANCE OUTCOME

In Hong Kong, H5N1 HPAI not only occurred in gallinaceous birds on farms and poultry markets, but was also seen in migratory in waterfowls and more recently other land-based wild birds. Besides the routine depopulation, cleaning, and disinfection procedures (DCDP), stringent biosecurity plans have been continuously revised for enhancement since 1997. Sentinels are placed in DCDP farms and no H5 AI exposure need to be demonstrated before chick placement on farms. The biosecurity plans are based on Hazard Analysis Critical Control Point (HACCP) principles to control the disease to enter the population and to minimize the impact if it enters the population (8, 12). The control points for the hazard are: movement control of infected animals and equipment (by requiring tests and certificates to avoid purchasing from potentially infected sources as carrier animals and infected birds in the incubation phase may appear healthy), wild bird and rodent control (by applying bird proofing sheds and removing spilt feed), access to pathogen-carrying aerosols (by separating farms and sheds with solid barriers or a distance of at least 500 m), and cross-contamination between species (by segregating species on farm, in transit and at markets). 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, and use of vaccines to increase resistance to the disease (8, 12). The use of vaccine is an additional control measure to aim at eradication of the disease.

The results of 2002-2003 AI vaccination field trial with Nobilis IA inac (H5N2 inactivated oil emulsion vaccine) results showed that the protective antibody responses were satisfactory, and there was no H5N1 infection detected using virus isolation and RT-PCR in any of the vaccinated flocks despite the outbreaks in the local park birds and poultry farms during the period (8). The vaccine was also shown to play a role in interrupting virus transmission in the face of an outbreak (5). The AI vaccination program then extended to all chicken farms and placed in the infected government recreation park (Kowloon Park) following the outbreak in 2002 has later encouraged a similar practice in other bird parks in HK (7, 8, 9). The combination vaccine, Nobilis IA+ND inac (H5N2 and ND inactivated oil emulsion vaccine) was introduced in 2004 and has been used since to control concurrent Newcastle disease (ND) which is an endemic disease and can have impact on AI control program. Vaccination and handling techniques, vaccine storage condition, farm management and practice, biosecurity, chicken house setup, ventilation, health status of the birds, immunosuppressive and other diseases, interpretation of serology in line with routine baseline comparison, and antigen detection are all important factors contributing to the success of AI vaccination. The vaccine efficacy studies against local and Asian isolates have also been done by different laboratories in Hong Kong (4), USA (10), Japan, Germany, the Netherlands, and USA (unpublished reports) in different period of times throughout these years. Encouragingly, commercial poultry farms that have adopted the same vaccination protocol continued to enjoy the free of HPAI H5N1 status based.

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AVIAN INFLUENZA H5N1 IN EUROPE - A GLIMPSE ON SOME REALITIES

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INTRODUCTION

Avian influenza (AI) H5N1 outbreaks in Asia and other countries have led to the imposition of import bans on poultry products from disease-affected countries. Consequently, the global market impact of these bans led to a progressive shortage of poultry meat, escalating world poultry prices, a sharp drop in global poultry trade, and trade diversion as countries try to buy product from disease free countries. The overall price impact on poultry prices in 2004 and 2005 has been additionally aggravated by shortages of other meats, particularly beef from North America (1).

THE SITUATION IN EUROPE

The 25 member states of the European Union count for about 13% of the world's poultry production and exports. France, the UK, Spain, Italy and Germany are the main production states and count together for roughly 2/3 of the EU's poultry production. While the EU imports approximately 700,000 tons of frozen fillets etc, poultry exports contribute significantly to the economy, too: the EU ships approximately one million tons of fresh, chilled or frozen poultry products, valued at over $1 billion euros, to more than 150 markets worldwide. The main markets are Russia, the Middle East, and developing countries, mainly on the African continent.

In Directive 92/40/EEC, the EU legislation defines the minimum control measures in the event of an outbreak of AI, and the aim is to reduce the risk that H5N1 spreads among poultry and other captive bird populations in the EU. All Member States have avian influenza contingency plans in place in case of an outbreak and the EU works closely with international partners such as the World Organization for Animal Health (OIE) and the UN Food and Agriculture Organization (FAO) on this issue (2).

To have a valid tool to deal with cases of low pathogenic avian influenza, a new directive 2005/94/EC adopted in December 2005 includes new measures such as preventive vaccination. This new legislation is in force, but Member States are obliged to implement it only as of 1 July 2007 (3).

Surveillance of sick and dead birds (wild and domestic) for highly pathogenic avian influenza (HPAI) is particularly strong. In 2006 this mechanism monitored the extension of the H5N1 virus from the East in wild birds including non-migratory species in 13 countries and some domestic poultry have also been found infected. Although surveys of healthy wild birds in the EU have so far indicated that H5N1 infection is very rare, there is a continuing risk of H5N1 appearing in parts of eastern Europe, especially around the Danube Basin.

After early in 2006 infections with avian influenza H5N1 were detected in several parts of Europe, both the various governments' sides as well as the respective populations responded. Within days, consumers' reactions had a massive impact on the poultry industry in Europe. Consumption of poultry meat dropped massively in Italy (4) and Greece, and France lost a significant portion of its exports.

EU exports over the first four months of 2006 were down 22%, mainly as a result of the partial and full bans on chicken from France, after its AI outbreak in a commercial turkey flock in February 2006. Due to the outbreaks of AI, total EU broiler production in 2006 was expected to be 4% lower than last year (about 8.025 million metric tons of broiler meat) but looks set to rebound in 2007. Chicken imports into the EU were expected to increase in 2006 by 15 % or more (5).

In the winter of 2005/06, it was bird flu (AI) which was occupying the thoughts of doctors, the media, and the public. A year later, the H5N1 virus is still around. Europe has seen no cases since the outbreak in Turkey, nor has Africa been hit by any major outbreaks since the beginning of the year. It is likely that the coming months will see more cases of birds infected with the H5N1 virus. But the experts' conclusion is that the chances it will mutate into a dangerous pandemic strain are no greater than they were this time last year (6).

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INTERNATIONAL COOPERATION ACTIVITIES TO SUPPORT DEVELOPING COUNTRIES IN MANAGING THE GLOBAL H5N1 AVIAN INFLUENZA CRISIS

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SUMMARY

Since 1997 highly pathogenic avian influenza (HPAI) H5N1 has spread to numerous countries, from Asia across East Europe to Africa, infecting not only a large number of poultry, but also causing an increasing number of human fatalities (153/258, WHO 30 November 2006). For these reasons and for the devastating effects of this disease on the poultry sector, international organizations such as WHO/OIE/FAO have worked together to establish a coordinated set of guidelines and action plans to combat the ongoing epidemic (3).

In this scenario, the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) in Padova, Italy, as an OIE/FAO reference laboratory for avian influenza (AI) was requested to increase its cooperation activities providing diagnostic assistance and technical consultancy to African, East European, and Asian countries.

The activities supporting beneficiary countries in the management of the H5N1 epidemic have been focused on:

1. Transfer of knowledge and technologies. Over the last two years IZSVe has provided training in applied diagnostic techniques for avian influenza to 19 veterinarians/technicians coming from Africa [8], Asia [3], South America [6], and Eastern Europe [2]. IZSVe has provided courses in serological, virological, and molecular diagnostic techniques. At the same time, IZSVe organized and performed eight training courses in the Central Veterinary Laboratories (CVL) of recipient countries. A total of 138 laboratory scientists and technicians coming from 62 countries attended these courses. Moreover, in order to standardize diagnostic procedures, protocols and standardized reference reagents complying with OIE and EU guidelines have been provided to all countries requesting them. In addition, a ring trial was performed at an international level to assess the capabilities of each laboratory in AI diagnosis.

2. Consultancy and diagnostic support. As an OIE/FAO reference laboratory for AI, IZSVe has been asked to confirm suspected HPAI and to advise the relevant authorities on how to confront the H5N1 crisis. More than 800 samples for confirmatory diagnosis of HPAI coming from INCO countries were tested in 2006 (2). Four laboratory assessment missions were performed to evaluate upgrading requirements in the CVL of Turkey, Mauritania, Kazakhstan, and Bulgaria. Concurrently, a consultancy was carried out in Vietnam to determine the efficacy of the national vaccination campaign. In addition, a vaccine scheme against H5N1 HPAI in ducks, with the South East Asian farming system in mind, was tested by IZSVe both under experimental and field conditions.

3. Strengthening laboratory networks. The training courses organized led to personal contacts between scientists and technicians working on AI. This type of networking is an immediate and efficacious tool in setting up collaborations and confronting an epidemic situation. This has been clearly demonstrated by the timely diagnosis of H5N1 in Western African countries as a result of initial contact between IZSVe and African staff through an AI workshop. IZSVe is also actively involved in promoting initiatives to share scientific information. An important example is the recent creation of the GISAID initiative (Global Initiative on Sharing Avian Influenza Data), an international collaborative effort on sequence and data sharing of AI (1). In collaboration with FAO and OIE the IZSVe has initiated the OFFLU network (www.offlu.net) which provides permanently updated information on diagnosis of AI, training opportunities, and the promotion of the exchange of viruses and reagents.

The importance of rapid detection and identification of H5N1 HPAI to control the disease in poultry, to limit virus circulation in susceptible species, and finally to avoid human infection, is well recognized. Veterinary laboratories, together with veterinary services, in each country should be capable of providing preliminary and differential diagnosis and laboratory confirmation of AI in a timely manner using standardized protocols and reagents. To achieve this goal, training of veterinary staff is compulsory.

Education and training should be dispensed taking into account a number of factors which include the expertise of the personnel being trained, the conditions of the laboratory where the trainee works, the availability and cost of reagents and consumables, and the funds available. With these factors in mind, the IZSVe's goal would be to train laboratory staff in a personalized manner and to monitor their activity for a significant period after training. This approach would avoid the wasting of resources and optimize the efforts of the cooperation projects.

Consultancy on laboratory infrastructures, equipment and rapid tests for the detection of avian influenza viruses and antibodies were the predominant requests from beneficiary countries. The main problems to be faced in these areas are:

• **The adaptation of standardized protocols to field conditions and non-conventional avian species.**

• **The availability of diagnostic reagents and kits in a timely and cost effective manner.** In some instances, the supply of equipment is not accompanied by the provision of specific reagents due to the absence of local providers or distributors, prohibitive cost and the unavailability of the cold-chain to guarantee reagent preservation.

• **The development and application of standardized guidelines to facilitate sample collection and shipment to Central and Reference Laboratories.** In many instances, local veterinarians and paraveterinary staff do not have sufficient equipment or training to enabling them to collect samples in the correct manner and have them delivered to the diagnostic laboratory. This represents a critical point, since in many countries prompt clinical disease recognition, sample collection and shipment to international reference laboratories are the only possibilities presently available for the testing and confirmation of AI.

The ongoing H5N1 epidemic is unprecedented in its spread. Therefore, it is essential that international organizations and reference laboratories fully support the implementation of veterinary services and diagnostic laboratories in order to make the control of HPAI affordable to developing areas. Funds and technical support should be distributed in an organized and beneficial manner, taking account of logistic and technical problems that may be encountered in different countries.

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VIRAL INFLUENCE ON THE PREVALENCE OF AIRSACCULITIS IN BROILER SLAUGHTER PLANTS IN QUEBEC, CANADA

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INTRODUCTION

In the spring and summer of 2004, an unusually high prevalence of broiler chicken carcasses condemned for airsacculitis was observed in Québec slaughter plants. A statistical analysis of data from participating slaughter houses revealed that airsacculitis condemnation rates were higher in May and June 2004 when compared to the previous year and mostly affected three regions. Males were also at greater risk of developing airsacculitis than females. An examination of laboratory results provided by Quebec poultry veterinarians revealed the presence of infectious bronchitis and infectious bursal disease in affected flocks. A paired case-control study was undertaken to identify risk factors and viral strains associated with the presence of high airsacculitis condemnations in broiler flocks. This abstract reports the results for the viral portion of the project.

MATERIAL AND METHODS

Study design. A paired case-control study design was conducted from May 2005 to February 2006. The study population was limited to male broiler chicken flocks from the three affected regions. Thirty flocks with high airsacculitis prevalence were compared with 30 flocks with low prevalence. The flock was the unit of concern and analysis.

Definition and selection of cases and controls. Carcasses condemned for airsacculitis based on criteria established by the Canadian Food Inspection Agency (CFIA) and carcasses reconditioned for minor airsacculitis lesions were included in the study. The incidence for airsacculitis was expressed as the number of carcasses condemned and reconditioned divided by the total number of broilers in the flock. Flock was defined as a group of broilers placed on a farm and processed together. Flocks with high airsacculitis prevalence at slaughter were considered cases. Each case was matched with a low prevalence airsacculitis flock slaughtered on the same day and coming from the same region as the case.

Tissue sampling. For each case and control flocks, lung, kidney, and cecal tonsils were collected from ten carcasses presenting airsacculitis lesions. All samples were identified and stored at -70°C until processed for isolation of infectious bronchitis virus (IBV), infectious bursal disease (IBD), Newcastle disease virus (NDV), adenovirus (AV), and reovirus (REO). Isolated viruses were tested by PCR amplification (Polymerase Chain Reaction) followed by restriction analysis or sequencing. All viral analyses were performed in Dr. Davor Ojkic's laboratory at the Animal Health Laboratories, University of Guelph, Guelph, Ontario, Canada.

Data collection. Data collected from abattoir included for each flock, name and address, total number of birds in the flock, and condemnation data including number of carcasses condemned and reconditioned for airsacculitis. A questionnaire regarding vaccination, management, and biosecurity measures was filled out by the owner at the time of a visit of the broiler house.

Statistical analysis. A McNemar test was performed to verify the significance between viral isolation and airsacculitis condemnation rate (SAS, version 9.0 Statistical Analysis Systems Inc., Cary, NC. Institute, 2005).

RESULTS

This study was done between May 2005 and February 2006. Of the 60 flocks initially included in the study, one case was not properly matched. Therefore, this pair was removed from the study, leaving a revised total of 58 sampled flocks (29 cases with high and 29 controls with low incidence of airsacculitis lesions at the abattoir).

Adenoviruses were the most prevalent viruses in both case and control flocks followed by infectious bursal disease (IBD) virus (Table 1). Infectious bronchitis virus (IBV) was detected in 23 of the 29 case flocks and was found to be significantly associated with a high prevalence of airsacculitis (Table 1).

Viral strain identification revealed that NC171 (n=36) was the most prevalent IBD strain in the 58 sampled flocks. Some 586 IBD (n=5) and Del-E IBD strains (n=5) were also identified. An IBV strain first

isolated in Quebec, QuMv, was the most prevalent (n=23) while Conn-CU510 and CV82792 were also isolated in five and two flocks respectively.

DISCUSSION

One of the objectives of our study was to identify viral strains associated with the presence of high airsacculitis condemnations in broilers. The QuMv IBV strain was the virus most significantly associated with such condemnations. This strain was first characterized by Smati *et al*. in 2002 (1) and remains the most prevalent IBV strain in the province of Quebec (Dr. Ojkic, AHL, OMAFRA, Personal communication). Unfortunately, since QuMv IBV strain is considered a new phylogenetic cluster, available commercial vaccines do not appear to clinically protect flocks. Further studies are warranted.

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Table 1. Number and proportion of viral strains isolated from case (n=29) and control (n=29) broiler chicken flocks collected in Quebec slaughter plants between May 2005 and February 2006.

Virus	Case	Control	*P* value for the McNemar test
Infectious bronchitis	79.3% (23/29)	31.0% (9/29)	p= 0.0012
Infectious bursal disease	86.2% (25/29)	58.6% (17/29)	p = 0.061
Adenovirus	79.3% 23/29	89.6% 26/29	p= 0.50
Newcastle disease	6.9% 2/29	3.4% 1/29	
Reovirus	24.1% 7/29	31.3% 9/29	p = 0.78

ADAPTIVE MOLECULAR EVOLUTION IN THE ARKANSAS SEROTYPE OF INFECTIOUS BRONCHITIS VIRUS

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INTRODUCTION

Avian infectious bronchitis is a highly contagious upper respiratory disease in chickens that is worldwide in distribution. It is caused by a coronavirus that is extremely difficult to control because infection or vaccination with one type of the virus will not cross-protect against another. Currently, the best strategy for control of this disease is the use of modified live infectious bronchitis virus (IBV) vaccines. Different types of IBV vaccines include multiple serotypes and variants of the virus. Coronaviruses have been shown, both experimentally and in nature, to undergo genetic mutations, from a polymerase lacking proofreading activity, and recombination, by a genomic template switching mechanism. Viral molecular evolution is the process whereby genetic mutations and exchange of genetic information among coronaviruses, leads to the emergence of new viruses capable of infecting and

causing disease even in immunized animals. Molecular viral evolution can be measured by examining changes in the viral genome over time. Herein we examine the Darwinian evolution among the Arkansas type IBV genotype by comparing the synonymous (d_S) and non-synonymous (d_N) codon based distances in the S1 gene.

DATA MINING AND SEQUENCING

Nineteen different Arkansas S1 genes from viruses isolated over a 33-year period were analyzed. Viruses and sequence accession numbers in GenBank include Ark/CU-T2/93 AIU04739, Ark/15C/96 AF169859, Ark/99/73 IBBSPIKE, Ark/213/96 AF169860, Ark/1529/95 AF169856, Ark/1534/95 AF169857, Ark/1535/95 AF169858, Ark/DPI/81 AF006624, Ark/K2-6/04 AY790369, Ark/K434/01 AY790368, Ark/K545/02 AY790366, and Ark/PP14/91 M99483.

Viruses sequenced herein include Ark/GAV/93, Ark-like/0015/97, Ark-like/4207/97, Ark-like/3668/97, Ark/MWJ51398/06, Ark/MWJ52052/06, and Ark/MWJ52930/06. For sequencing, viral RNA was extracted from the phenol-inactivated viruses (High Pure RNA Isolation Kit, Roche Diagnostics, Penzberg, Germany) and used as template in the reverse transcriptase-polymerase chain reaction (RT-PCR, Titan One Tube RT-PCR System, Roche Diagnostics). The amplified DNA was electrophoresed on a 1% agarose gel, cut out of the gel and purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA). Primers used in the RT-PCR reaction were previously reported (1). Sequencing was conducted at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (2). The Clustal V method of alignment was conducted and phylogeny reconstruction was completed using the Neighbor-Joining, Nei-Gojobori method (p-distance). Phylogenetic trees were generated with 1000 bootstrap replicates in heuristic search. Evolutionary distances were determined by the Z-test of positive selection ($d_N > d_S$).

RESULTS AND DISCUSSION

In this study, nineteen S1 genes from the ATG start site to, and including, the cleavage site were examined for phylogeny and molecular genetic evolution. Phylogeny reconstruction using the Nei-Gojobori (p-distance) analysis showed that three 2006 isolates and one 1997 isolate grouped closely with the vaccine strain Ark/DPI/81 indicating that these viruses are likely vaccine in origin but have undergone some minimal genetic drift. A second group comprises strains isolated between 1995 and 1997, and a third group is formed by isolates from Korea. Examining the evolutionary distances for the non-synonymous and synonymous changes in the spike gene of all of the viruses studied, showed that indeed the Arkansas viruses are evolving. The evolutionary data shows a common ancestor, Ark/99/73 isolate, which is the oldest known Arkansas strain. Darwinian molecular evolution that was likely influenced by vaccination is occurring between groups but not within groups. Previously we reported that the GA98 IBVs are evolving and likely arose from the DE072 type virus (3). In this study we show that the Arkansas type IBVs are also evolving. Past experience indicates that a new type of IBV will arise from the evolving Arkansas viruses; what is unclear is when that will occur.

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MOLECULAR CHARACTERISTICS OF TWO IBV SEROTYPES AFTER 10 AND 100 PASSAGES *IN OVO*, RESIDUAL PATHOGENICITY, AND IMMUNOGENICITY OF ATTENUATED VIRUS

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SUMMARY

The authors describe the characterization of two IBV serotypes isolated in Italy with regard to the molecular structure of a portion of hypervariable tract at level of few (6-9) and 100 embryo passages and the immunogenic activity. No (*AZ 40/05*) or only one (*AZ 27/98*) change on 127 amino acids, without apparent loss of immunogenicity after 100 passages, were observed.

INTRODUCTION

Infectious bronchitis virus (IBV) is considered as an antigenically very variable virus, unlike many other known coronavirus of the various animals and humans. In fact, from the first discovery in 1936 (1), IBV exists in over 60 different serotypes or variants, which are continuously evolving and increasing, apparently more in North-America and Europe (5, 7, 9, 16, 17, 19) than in other continents (8, 10, 11, 12). It follows that the control of the disease sometime results quite complex, particularly in long-life birds, even if, with the use of at least two serotypes, the range of protection can be remarkably extended.

Several isolates of IBV have been attenuated as from years 50s, above all by serial (40 to 120) embryo passages (6, 14, 15). Both the stability of virus attenuation and the conservation of antigenicity appear very important tools when the vaccine has to be used in the field, particularly in multi-aged farms. The use of mono- or polyvalent inactivated vaccines in oil emulsion before to enter in lay would induce a long lasting immunity.

Aim of the present paper is to report on: 1) possible variation in nucleotide and, consequently, in amino acid sequences in a part of S1 spike protein of two of the most common IBV strains recently spread in Europe, submitted to low and high *in ovo* passage, in order to evidence eventual differences; and 2) residual pathogenicity and immunogenicity of such attenuated virus strains.

MATERIALS AND METHODS

Pathogenicity and immunogenicity tests. SPF chicks, about three weeks old, were used, which in part were delivered via eye-nasal-drop with viruses of different passages of:

AZ-27/98 serotype:
Group *A*: no. 20 birds with $10^{3,5}$ EID_{50} of 9th passage;
B: no. 20 birds with 10^{4} EID_{50} of 50th passage;
C: no. 20 birds with 10^{4} EID_{50} of 100th passage;
D: no. 15 birds of control.

AZ-40/05 serotype:
Group *E*: no. 20 birds with 10^{4} EID_{50} of 6th passage;
F: no. 20 birds with $10^{4.5}$ EID_{50} of 100th passage;
G: no. 15 birds of control.

Examination procedures. All birds in experiment were constantly controlled for general, respiratory and enteric symptoms for at least 10 days after vaccination and also after challenge. Four days after administration of vaccine, four birds per group were humanely sacrificed; tracheas and sometimes kidneys and intestines were collected; part of tissues were fixed in 10% formalin in saline for histology, part were homogenized, single tissue and bird, suspended in PBS with antibiotics for virus reisolation in 10-day-old embryonated egg (one to three serial passages) in groups each of three eggs.

Challenge. Three weeks post vaccination 12 birds of group B, C, and F and five birds of group D and G were challenged, via eye-nasal-drop, with 10^{6} EID_{50} of homologous or heterologous *AZ-27/98 or AZ-40/05* isolates at 9th embryo passage; five days after challenge five to seven birds of each group were killed and tracheas collected for eventual reisolation of virus and for histological examination.

Serology. Three weeks after vaccination and before challenge, the sera from four birds per group were collected for titration of antibody by virus-

neutralization test, using β-method, variant virus-constant serum (18); neutralization indexes (INs) were calculated according to the method of Reed & Muench.

Molecular characterization. IBV strains: *AZ-27/98*, 10th, 85th and 100th passage, *AZ-40/05*, 10th, 60th and 100th passage in embryo isolated respectively in 1998 and 2005; the material for sequentiation is represented by allantoic fluid of infected embryonated eggs.

RT-PCR tests were performed according to the described methods (2, 4), as follows:

Extraction of RNA from allantoic fluids, purification by the method of Chomezynski and Sacchi (4) and storage at -20°C;

RNA detection by *RT-PCR* assay using primers: *XCE1+*(5' CTC TAT AAA CAC CTT ACA 3') plus *XCE2─* (5' CTC TAT AAA CAC YTT ACA 3') or *IBP1*(5' CAA TTA ATT TGG ACC TTA TCCA 3'), able to amplify respectively a fragment of 456 bp or 1100 bp of hypervariable region of S1 spike protein;

Amplified fragments analysis by 1.7 agarose gel electrophoresis, stained with ethidium bromide and observed with an ultraviolet transilluminator; and

Sequencing of various strains performed on PCR product directly after gel purification with Qiaquick gel extraction kit (QUIAGEN) with primers *XCE1+* and *XCE2-* and *IBP1*, by means of a Big Dye Terminator DNA Sequencing Kit on ABI Prism 3130 Genetic Analyzer (Applied Biosystem, Foster City, CA).Comparative analysis of nucleotide and deduced amino acid sequences was carried out with Clustal W Package of DNA Star (MegAlign v.5 2001; DNA Star inc., Madison, WI). A molecular weight standard was added and used to determine the size of cDNA fragments.

Histology. The specimens were stained with the standard method of hematoxilin-eosin.

RESULTS

Molecular characterization. The comparative analysis of 152 amino acids (from *aa* 235 to *aa* 387, numbering referred to M41 strain) representing a part of hypervariable portion of S1 spike protein of IBV, between low (10th) and high (100th) *in ovo* passages of the *AZ-27/98* and *AZ-40/05* strains, showed an *aa* substitution in position 341 (residue in leucine-phenilalanine) only in the first strain. That could explain the good conservation of antigenic characteristics of both viruses.

Pathogenicity and immunogenicity tests. The trials to control the residual pathogenicity and immunogenicity of the considered virus strains, after 50 and 100 passages *in ovo* demonstrated that:

1) chicks receiving by eye-nasal-drop also EID_{50} $10^{4.5}$ log_{10} of virus, showed in the following 10 days, very light respiratory symptoms in few birds (*AZ-27/98*) or no troubles at all(*AZ-40/05*); vaccinal virus was reisolated after four to six days from all examined tracheas of inoculated chicks, but not of controls; the control chicks, inoculated with low passages of virus by the same route, showed malaise, respiratory, and enteric symptoms from two to three days after, until 10 days; and

2) apparent loss of the first and the maintenance of the second feature, i.e., the development of remarkable antibody titer (IN=4.2-5.0 log_{10}) three weeks after vaccination and resistance to a homologous virus (100%); that obviously means an optimal immunity; on the contrary the cross-immunity between the two serotypes resulted to be only partial (about 25%).The results are reported in Table 1.

Histology. Tracheas of birds delivered with *AZ-27/98* strain at 50th and 100th passage and with *AZ 40/05* strain at 100th passage showed very few or no microscopic lesions on the mucosa. Tracheas of birds vaccinated and challenged with pathogenic low passage of homologous virus showed no microscopic lesions, whereas the birds infected with heterologous virus (3 on 4) and all control birds showed severe lesions on the mucosa (depithelization) and submucosa (cell infiltration).

DISCUSSION

The non-significant differences in sequences of hypervariable portion of S1 spike protein, comparing low and high embryo passages of both viruses, *AZ-27/98* (1 *aa*) and *AZ-40/05* (none) serotypes, explain the good conservation of antigenic characteristics, consequently the respective high antibody response. So about one hundred passages *in ovo* wouldn't modify substantially the immunogenicity of such IBV strains. However, a lesser ability to replicate *in vivo* could occur; that should be verified better. Besides, also H120 strain (120 passages), deriving from Massachusetts serotype, universally used to vaccinate birds of all ages against IB, has the same sequence in the same portion of H52 strain (52 passages). No differences were previously observed also in *AZ-23/74* serotype (20). A difference of only one *aa* (residue in serine-alanine position 283) in S1 spike protein of UK-4/91strain after various (no mentioned) passages were also reported; such puntiform change was apparently not associated with the pathogenicity of the virus (3). More substitutions of *aa* (2 to 5) in the complete sequence of S1 spike protein have been recently reported between low (7th) and high (76th) *in ovo* passage of some IBV isolates in Taiwan (13), but if the

numbering is corresponding, not in the same sequence portion of S1 spike protein examined in our strains.

The trials to define the residual pathogenicity and the immunogenicity of the strains *AZ-27/98* at 50th and 100th passage and *AZ-40/05* at 100th passage demonstrated the lack of the first and the maintenance of the second characteristic, that is the development of a remarkable antibody reaction but, mostly, the resistance to the challenge, after all the immunity. The virological results were confirmed by histological ones, which showed no or very few microscopy lesions in tracheas of bird delivered with 100th passage of both considered strains of IBV, also after challenge. The stability of attenuation and the changes, which occur to the genome of IBV, are aspects that, up today, have not received as much attention as they should. So they have to be studied more in depth in order to get out of unpleasant mishaps in field, particularly in multi-aged farm and in areas with crowded breeding, where the vaccinal virus could regain a certain degree of pathogenicity, if the attenuation is not very stable.

In conclusion, the attenuation and the antigenic stability of the virus appear to be of paramount importance in order to licence a live attenuated IBV vaccine (14, 15). Further studies are in progress to better characterize the variations in other parts of the genoma between and respective attenuated virus strains and to screen the best serotypes combination of IBV for vaccination.

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Table 1. Inoculation, via oculonasal route, with IBV AZ-27/98 and AZ-40/05 strains (100 embryo passages) and homologous or heterologous challenge with the same pathogenic strains at 11th passage.

Group	No. chicks	Vaccine type	Reaction (symptoms)	Vacc.virus reisolation after 4 days	Antibody VN after 3 wk (NI)	Chall. 3 wk after vacci nation	Reisolate virus chall	Respirat. symptoms
1	20	AZ-27/98	Very light in some birds	4/4	4.2 (4.0-4.7)	AZ-27/98	0/4	---- --- ±±± --
						AZ-40/05	3/4	
2	20	AZ-40/05	no	4/4	5.0 (4.5-5.5)	AZ-27/98	3/4	+++± -- ---- --
						AZ-40/05	0/4	
3	15+15	-	no	0/4	< 2.0	AZ-27/98	4/4	+++++
						AZ-40/05	4/4	+++++

CHICKEN HARDERIAN GLAND AND LACHRYMAL FLUID: A SOURCE FOR INFECTIOUS BRONCHITIS VIRUS AND SPECIFIC LYMPHOCYTES

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SUMMARY

High concentrations of infectious bronchitis virus (IBV) genomes can be detected by RT-PCR in lachrymal fluid samples from IBV infected chickens. To demonstrate that IBV contained in lachrymal fluids is infectious, and not neutralized by antibodies or inactivated by other components of tears, we collected tears from SPF chickens eight days after ocular inoculation with an Ark-type IBV field isolate. Presence of IBV in each sample was verified by RT-PCR. Samples were pooled, diluted, and inoculated ocularly into naïve SPF chickens. Additionally, the pooled, diluted tear samples were frozen and thawed once before inoculation into another group of chickens to determine whether virus in tears stored frozen could be used to transfer IBV. Our results showed that both fresh tears and tears subjected to one freezing and thawing cycle successfully reproduced the disease in susceptible chickens.

We monitored the immune responses to IBV in the Harderian gland of SPF chickens infected with IBV by the oculonasal route and compared these responses in immunocompetent IBV-infected chickens and chickens infected with IBV and immunosuppressive viruses CAV and/or IBDV. We analyzed CD4/CD8 T cell ratios in the Harderian glands by flow cytometry and found that CAV, but not IBDV, reduced the T helper / T cytotoxic cell ratio compared to chickens infected with IBV alone. The relative abundance of IgM+ B cells in the Harderian glands following exposure to IBV in combination with immunosuppressive viruses was reduced compared to chickens infected with IBV alone. We detected IBV-specific IgA spot-forming cells in the Harderian glands of IBV-infected chickens by ELISPOT assays. IBV-

specific IgA-secreting cells were reduced in chickens infected with immunosuppressive viruses. Thus, analyses of immune responses at this mucosal effector site indicate that immunosuppressive viruses reduce B cells and T helper cells in the Harderian glands, and predict a decrease in the functional mucosal immune response against IBV.

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

SELECTION OF VACCINE STRAINS FOR AVIAN INFLUENZA VACCINATION

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SUMMARY

With human influenza viruses it is clearly recognized that the vaccine seed strain used for both killed and modified live vaccines (cold adapted) must be closely matched to the circulating strain of influenza to provide adequate protection from exposure. A vast network of laboratories have been developed (World Health Organization Global Influenza Program) to routinely isolate and characterize influenza isolates from around the world. Selected viruses are sequenced and a smaller number are antigenically characterized to provide the information needed to select the vaccine seed strains to make recommendations for the vaccine for coming year (1). The human influenza vaccine seed selection is unique in the depth and scope that occurs to select a vaccine on a yearly basis.

The use of vaccination for avian influenza viruses for poultry has been increasing in recent years, but currently no unified program for vaccine selection exists for poultry vaccines. Avian influenza virus differs from human influenza primarily by the adaptation of the virus to be host specific. The same principles of antigenic drift are occurring with avian influenza as occurs with human influenza (2, 3). However, two important differences separate avian from human influenza. First, vaccination to avian influenza provides broader protection to highly pathogenic avian influenza infection as will be described in more detail below. The second major difference is that only two lineages of Type A human influenza currently circulate at any given time, H3N2 and H1N1. With avian influenza in poultry, many more subtypes of avian influenza infect poultry in any given year. Rarely, particular subtypes become endemic in poultry like H6N2 in California, H7N2 in the Northeast U.S., H9N2 in the Middle East and Asia, and of particular concern H5N1 in Asia, Europe and Africa. These endemic outbreaks, although likely from a single original source, as they have spread have developed in geographically isolated pockets. This has resulted in unique genetic and antigenic sublineages. For Asian H5N1 this has been described as three distinct antigenic clades (4). Because of these differences, the human vaccine selection program serves as only a limited model for what should be done in poultry medicine.

To understand the apparent contradictions between avian and human influenza, the differences in the biology of infection between the two must be understood. First is that HPAI is a mucosal and a systemic infection, but human influenza is a mucosal infection. For HPAI, viremia likely disseminates the virus systemically. If the viremia can be blocked, the systemic infection is prevented along with the severe pathology and clinical disease associated with systemic infection. The use of killed parenterally administered vaccines provides good serum antibody levels and this serum antibody appears to block viremia even when the vaccine is not well matched to the challenge strain of virus. Killed parenteral administered vaccines, however, do not provide high levels of antibody on the mucosal surface. Therefore our understanding of how vaccines behave are more analogous on the mucosal surface for human and avian influenza vaccines, and a poorly matched vaccine to challenge can result in clinical disease and high virus shedding.

What defines a good vaccine? We can use several criteria. The most commonly used method for evaluating vaccine seed strains with circulating field strains is with the hemagglutinin inhibition test. This test uses antibody produced from infection or vaccination of the target species and this antibody is compared with antigen of the field strain to measure levels of inhibition measured by dilution of the serum.

The antibody is also compared with itself as antigen to produce a standard. If the vaccine produces antibody that neutralizes itself at a dilution of 512 and neutralizes the field strain at the same level, it would be considered a well matched vaccine. However, if it only neutralizes the field strain at a level of dilution of 128 or lower (a four fold drop or greater in antibody specificity) it would be considered a poor antigenic match. For humans a four fold or greater drop in protection is a strong indicator that the vaccine seed strain needs to be updated. The use of cross HI titers, where antibody and antigen from both vaccine and field strain are compared to each other can provide additional criteria of protection and this data can be applied with the Archetti and Horsfall formula to predict antigenic relationships (2). An alternative method of evaluating antigenic relatedness is the virus neutralization test in cell culture. The VN test and the HI tests typically give similar results, although the VN may be a better assessment of protection since it more closely resembles neutralization *in vivo*.

An alternative method of assessment is the sequence analysis of vaccine and field strains. It has become routine to sequence influenza viruses, particularly the hemagglutinin gene, which is the primary protective antigen for avian influenza. The putative amino acid sequence is commonly compared, and it has been shown to have some predicting power for levels of shedding from HPAI viruses. The closer the vaccine is to the challenge virus, the less virus will be shed (5). Sequence analysis, although useful, does have limitations. The protective antigenic epitopes for avian influenza are not uniformly spread over the hemagglutinin protein. It has been suggested that at least five protective antigenic regions occur for H5 influenza, and single point mutations in these epitopes can adversely affect protection. The comparison of overall amino acid similarity may suggest a closer antigenic relationship than actually occurs because of the positive selection at these antigenic sites. This appears to be occurring in both the Mexican H5N2 outbreak (2) and in Asia with H5N1. The HPAI H5N1 strain in Asia has been classified into three antigenic clades with HI differences of eight-fold being common, but these clades genetically are only 5-6% different at the amino acid level (4). Currently, we don't have a model for evaluating just the sequence of the antigenic regions in predicting antigenic relatedness.

The most comprehensive way to measure vaccine effectiveness is through animal challenge models. The use of animal challenge is not performed routinely because of the high cost. The observation of clinical signs after challenge with a virulent virus is an important measure of protection, but the more quantitative measure of protection is determining viral shedding. A vaccine that produces sufficient levels of serum antibody can block the viremic stage of disease and reduce or prevent clinical evidence of infection. The measurement of virus shedding from mucosal surfaces, which is more important to virus transmission, provides a more discriminating method of evaluating protection (5). Levels of virus shedding after challenge can also be used for comparison of vaccine efficacy for both low pathogenic and highly pathogenic AI challenge.

The criteria for selecting a vaccine seed strain or gene insert for a recombinant vectored vaccine should include both antigenic and sequence analysis. The use of challenge studies should also be used, but for practical reasons will not be used commonly. Suggested criteria for selection are vaccines to have less than a four-fold difference in HI titer to field vaccine, less than 5% aa sequence differences, and in challenge studies result in at least a two log reduction in virus shedding compared to unvaccinated control birds. Additional research and experience are needed to develop more a more comprehensive strategy for vaccine selection. In addition the appreciation of government and regulatory bodies of the need for vaccine selection will be required.

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DEVELOPMENT OF LIVE ATTENUATED VACCINE AGAINST AVIAN INFLUENZA

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SUMMARY

Live-virus vaccines have distinct advantages over inactivated vaccines such as triggering mucosal immune responses and inducing a cell-mediated immunity, which may give the animal a more cross-protective and longer lasting immunity. Furthermore, live-virus vaccines can be administered by aerosol – a distinct advantage over the more difficult and costly route of intramuscular injection using needles. However, the use of live influenza vaccines in poultry has never been seriously considered because of the potential for the virus to become established as an endemic infection. In addition, the likelihood that recombination with a circulating virus would create a new reassortant virus has also been a concern. However, recent advancements in biotechnology have posed the possibility of considering new vaccine approaches using attenuated live influenza virus. The influenza virus NS1 protein has several advantages as a target for attenuation (1). First, NS1 protein functions as an interferon antagonist and is thus directly associated with the pathogenicity of the virus. Second, because NS1 protein is not incorporated into virions, it does not presumably interfere with the structure of the virions. Therefore, alterations of this protein would not change the antigenicity of the influenza virus itself. Furthermore, NS1 is synthesized in large quantities in infected cells and is known to tolerate long insertions of foreign sequences.

From the TK/OR/71-del (H7N3) virus, we previously found that several variants with different sizes of the NS gene can be generated by serial passage of the virus in embryonating chicken eggs (2). In this study, we further pursue the identification of different NS genes and have found 20 different NS genes that have unique deletions in different regions of the NS gene to date. To biologically purify the variants that have different NS genes, we passaged the parent TK/OR/71-del virus in 7-, 10- and 14-day old embryonating eggs. Then, we conducted intensive screening of the derivative viruses by the plaque purification method in chicken embryo fibroblast (CEF) cells followed by RT-PCR and sequencing. To date, we obtained three biologically purified variants (D-del var1, var2, and var3). We passaged those selected variants at least five times in 10-day-old eggs and confirmed that those NS genes are stable and do not produce additional variants.

To create a H5 vaccine strain (since the selected variants are H7 subtype) that contains the selected NS gene, we utilized a traditional reassortment method. Briefly, D-del var1 and TK/WI/68 (H5N9) viruses were co-infected into 10-day-old embryonating eggs for reassortment. After 48 hrs of co-infection, infectious allantoic fluid was harvested, followed by intensive plaque purification of derivatives in CEF cells. Individual clones were examined for their gene composition by RT-PCR and sequencing. We obtained a H5-D-del-v1 variant which has the NS gene of D-del var1 and other remaining genes of TK/WI/68 virus. Creation of H5 vaccine strains that contain other NS genes (NS gene from D-del var2 and var3) is ongoing.

We tested the pathogenicity of selected variants after inoculating eggs on the 17th day of embryonation. We inoculated 10 embryos each with $10^{1.0}$, $10^{2.0}$, and $10^{3.0}$ EID_{50}/0.1mL of three different selected variants (D-del var1, -var2, and -var3), H5-D-del-1 reassortant, and wild type H7 and H5 virus for comparison, respectively. At three days post-infection, we collected lung tissue for virus isolation and trachea, lung, spleen, bursa, kidney, and intestine for histopathology and immunohistochemistry. All NS gene variants replicated 1 or 2 log level lower ($10^{1.5}$ – $10^{3.6}$ EID_{50}/0.1mL) than the wild type viruses in the lung and all the $10^{1.0}$ and $10^{2.0}$ EID_{50}/0.1mL dose infected eggs showed no histopathologic lesions. We did not observe intensive IHC staining in different tissues other than lung and some connective tissues which may mainly be due to the lower titer of virus replication in those tissues. More intensive study using a large number of eggs will be conducted to examine the hatchability and to assess the potential of each variant as an *in ovo* vaccine candidate.

We expect that these naturally selected NS1 deletion variants will be useful in the development of live influenza vaccines both in their current state and with further modification of the NS1 protein. Furthermore, a deletion in the NS1 protein can also be useful as a negative marker for the DIVA (Differentiating Infected from Vaccinated Animals)

approach. Vaccines can be administered to chicken eggs during later stages of embryonation. We expect that in the future, an attenuated replication-defective virus capable of inducing strong immune responses could have potential as an *in ovo* vaccine that would not produce any infectious virus in hatched chicks.

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INACTIVATED MEXICAN H5N2 AVIAN INFLUENZA VACCINE PROTECT CHICKENS FROM THE ASIATIC HIGHLY PATHOGENIC H5N1 AVIAN INFLUENZA VIRUS

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SUMMARY

A laboratory trial was carried out in Japan by the Research Institute for Animal Science in Biochemistry and Toxicology (RIAS) and the National Institute of Animal Health (NIAH), to determine the efficacy of a killed avian influenza virus (AIV) vaccine subtype H5N2 produced by Avimex in Mexico with the strain A/chicken/Mexico/232/94/CPA of low pathogenicity (LP) against the challenge with an Asiatic highly pathogenic (HP) AIV subtype H5N1 (Japanese Yamaguchi strain).

SPF birds were vaccinated twice at 10 and 45 days of age. Four weeks post last vaccination, all birds were challenged intranasally with 0.1 mL of 106EID50 of the Yamaguchi strain.

Results derived from this limited experiment indicated that all vaccinated birds survived without showing any clinical signs, and without reisolation of the HPAIV in swab samples taken from trachea or cloaca at two and four days post challenge (PC), but also from internal main organs (brain, bursa of Fabricius and spleen) in a second vaccinated group in samples taken 4 days PC.

Differences from 3 to 5 log 2 in HI titers were found depending on the antigen tested (Mexican CPA-PRONABIVE '94 strain or Japanese Yamaguchi strain). Minimum HI antibody titers preventing infection were 1:40 and 1:10 for CPA and Yamaguchi antigens, respectively.

In the control group, all birds died within four days PC, with a mean death time estimated in 2.9 days. AIV was recovered in a high rate in all samples tested at four days PC from trachea and cloaca.

It is concluded that the Avimex Mexican vaccine elaborated with the subtype H5N2 is highly effective to prevent the Asiatic H5N1-HPAIV infection.

A NOVEL ENGINEERED LIVE VIRAL VACCINE AGAINST NEWCASTLE DISEASE AND AVIAN INFLUENZA SUBTYPE H5

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A novel Avimex® experimental recombinant live virus vaccine containing a Newcastle disease virus as a vector (lentogenic vaccinal strain) with the insertion of the HA gene of avian influenza virus subtype H5 was tested in high security units (level 3).

SPF birds were vaccinated at 10 days of age by eyedrop in the conjunctival sac (one single drop of 0.03 mL containing $10^{5.48}$ $CEID_{50}$). Another group remained unvaccinated. Twenty-one days later, vaccinated and unvaccinated birds were divided into two groups in separate units. One group was challenged with a Mexican velogenic Newcastle disease virus (VNDV) strain Chimalhuacán, with 0.2 mL of a $10^{6.0}$ $CEID_{50}$/mL inoculum by the intramuscular route. The second group was challenged with a Mexican highly pathogenic avian influenza virus (HPAIV) strain A/Chicken/Querétaro/14588-19/95 (H5N2), with 0.3 mL of a $10^{7.0}$ $CEID_{50}$/mL inoculum applied directly into the eyes and nostrils.

Results indicate that during the fourteen days period post challenge, 100% of unvaccinated-infected birds died when challenged with VNDV and 95% when challenged with the HPAIV H5N2. Vaccinated birds were fully protected (100%) against mortality when challenged with the VNDV and 90% were protected when challenged with the HPAIV H5N2.

The experimental live vaccine can play an important role when used as a complement to actual avian influenza killed vaccine programs in reducing virus shedding and raising the threshold for infection and transmission.

It is concluded that the experimental recombinant vaccine, expressing dual specificity against AIV and NDV, protects immunized birds when challenged with any of both pathogens.

VACCINATION AGAINST H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA: SOME QUESTIONS TO BE ADDRESSED

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INTRODUCTION

It is now more and more accepted that the panzootic of highly pathogenic avian influenza (HPAI) due to Asian H5N1 HPAI virus (HPAIV) is predominantly a problem of animal health, capable to generate severe damages to the poultry industry that are sociologically and financially of strong importance. Hence any action plan implemented to control the disease must aim at 1) keeping the poultry business running in affected areas, 2) preventing the spreading to not affected areas, and 3) eradicating the disease.

Very soon after the occurrence of the first breaks, the possible use of vaccines has given rise to controversy mainly based on somewhat academic and theoretical arguments when proper and coordinated faster application could have been of great help.

It was loudly said that vaccination would be an easy way to go that would reveal the incompetence of local veterinary authorities, and would be a major mistake since it would mask the circulation of the virus without preventing it. It was also said that vaccination could induce antigenic variations of the field virus and consequently end up with a reduced efficacy. It was even said that vaccination of poultry would ease the spread to humans, and possibly the emergence of the pandemia, and, as a summary, using vaccines against H5N1 HPAI would be the last thing to do.

And until now, these theories have kept going, leading to some absurd situations where vaccination is

largely used in the field although not officially recognized, that is to say, not organized and not controlled.

But it was nothing new to say that veterinary vaccines, including AI vaccines, are not perfect and in the vast majority of the cases, not capable just by themselves, to perfectly control a disease. However, they are widely used and any professional from the poultry industry would say that, in many cases, it is just not possible to work without them. Vaccines are just tools with potentialities and limits and as such need to be quite well known, well understood, and well applied, to get benefit from most of it. They can be very useful to control and eradicate AI and there are regions or countries where, at this time, poultry producers cannot economically survive without it.

The objective of this paper is to review some key points that need to be kept in mind when the use of vaccines against AI is under discussion.

ABOUT AI VACCINES

A surprisingly good clinical and anti-infective efficacy. Surprisingly, under laboratory conditions, classical inactivated AI vaccines have revealed extremely efficacious in preventing clinical signs and mortality in case of virulent challenge. Protection rates of 100% against very severe challenge are commonly seen. In some experiments that we have conducted, this was observed even when antigen concentration was significantly reduced. This very good clinical efficacy of the vaccines goes together with a very good anti-infective activity, if a proper dosage of antigen is administered. Re-excretion of the challenge virus through tracheal and cloacal routes is strongly reduced, considering both the amount of virus shed, and the duration of the shedding. It has also been shown that a higher dose of AIV was necessary to infect vaccinated chickens in comparison with not vaccinated.

Such outstanding performances are not common for poultry vaccines. In similar challenge conditions, they are never observed with inactivated ND (Newcastle disease) vaccines for example.

These are strong argument in favor of the use of AI vaccines: 1) to calm down a recently appeared and spreading epizootic (ring vaccination), and give more time to organize the control; 2) to protect any highly densely populated susceptible poultry area where any break lead to rapid spreading of the disease; and 3) to look at vaccination as a possible way to eradication in countries where AI is enzootic, provided a proper serological monitoring (DIVA) and action plan is designed.

However, it must be kept in mind that these intrinsic properties of classical AI vaccines are very much dependent on the quality of the vaccine as well as on the practical application.

The importance of the right vaccine virus strain. It is generally accepted that any AI vaccine is efficacious if the hemaglutinin (H) of the vaccine virus is of the same subtype as the one of the challenging virus. Similarly, virulence of the vaccine strain, before inactivation, is seen as of no importance regarding quality of induced immunity. In other words, any vaccine based on low pathogenic H5N2 or H5N9 AIV strains would induce consistent immunity against challenge with highly pathogenic Asian H5N1.

However, it is commonly said, in some affected countries, that vaccines including exactly the same H than the one of the field strain are more efficacious. This would mean that the best protection would be achieved by vaccines including the H5 of the HP Asian H5N1 AIV. This is very much in line with what has been reported in Mexico regarding the antigenic drift of the field H5N2 LPAIV and the necessary adaptation of the vaccine strains to keep good level of efficacy.

Although marketing and commercial issues are not independent from it, this argument may be relevant. But field experience tends to demonstrate that quality of vaccine is the first point to address, far before talking about the homology of the vaccine virus with the field virus.

The importance of the quality of the vaccine. The efficacy of any vaccine, and this includes AI vaccines as well, is very much dependent on the quality of the product as well as the quality of the manufacturing process and quality control procedures. The antigen concentration appears to be a key issue. Experiments we have conducted have demonstrated that reduction of the antigen content leads to a reduction of the capacity of the vaccine to prevent the shedding although antibody response to vaccination remains almost identical (within a certain range of concentrations). In other words, it seems that monitoring of antibody response in the fields is useful to check the quality of the vaccination but is not a very accurate and sensitive way to evaluate the level of protection.

The quality of the vaccine must be considered through the antigen content but also through the quality of the manufacturing process that is the only way to ensure the consistency of the product. Several criteria are of key importance including the inactivating agent, the possible residual presence of active formaldehyde that would progressively deteriorate the antigens, and the stability of the emulsion.

Because of this, it has been somewhat disappointing for serious vaccine producers to see all the commercially available vaccines grouped together on some official list, and because of this providing all

of them with some respectability, with no distinction or no warning regarding the possible variations in quality of product or production. To see H5 AI vaccine on the label is not sufficient to ensure any efficacy.

The problem of the necessary injection. At this time, all (almost all) types of AI vaccines that are commercially available (classical inactivated – recombinant Fowl Pox) need to be injected and are deprived with spreading capacities. For these reasons, the quality of the vaccines application is crucial since all non injected chickens won't be protected, and incompletely injected chicks will be poorly protected.

Individual injection of vaccines to large or very large numbers of birds is very much energy and time consuming and in practical very rarely well done. In reality, although quite astonishing, when proper checking is done using post-vaccination necropsy (residue of oil at the site of injection) or serological testing, it is not uncommon to see as much as 20% or 30% or even more of chickens that were not injected.

Thus, in practice, any prevention program based on vaccination should include training, motivation, and checking of the vaccination crews.

It is also important to keep in mind that vaccination crews must also be regarded as potential vectors of the disease, and to stress the importance of education and detailed procedures.

The unknown effects of maternally derived antibodies (MDA). In some countries, AI vaccines have been used for several months or years, including in breeding parent stocks, and consequently, day-old chicks are now (or will be) provided with specific maternally derived antibodies (MDA) against AIV. Taking other diseases in reference, we know that this is probably hampering in a significant manner the efficacy of the vaccination, but we do not know to which extend. Designing a vaccination program in chicks that are MDA free is quite simple, but doing it in MDA positive chicks is much more complicated since the level of MDA also needs to be taken into account. There is an obvious need for some practical research in this field.

Unfortunately, this issue will not be solved by the availability of a NDV-AI recombinant vaccine that is already (or will be) used in some countries. MDA against NDV (ND Virus), which are commonly found in young chickens in the majority of the countries, will for sure interfere with this vaccine. Interference of NDV MDA with the vector (NDV) will replace the problem of interference of AIV MDA with the classical AI vaccine.

Questions about the program of vaccination. Long living birds need to be revaccinated. In countries where AI has become enzootic, they are considered as more likely to be affected, and are the ones that are most commonly submitted to vaccination. However, either on an individual or on a flock basis, duration of immunity induced by available vaccines is not long enough to cover for sure the whole production period. And unfortunately, we have little solid information regarding the frequency of the vaccinations necessary to keep the layers protected. In affected areas, some breeders/layers flocks do receive up to five to six injections as apparently dictated by field experience, but no sound guidelines are actually available.

This is also another area where applied research would be worth to conduct.

ABOUT THE ENVIRONMENT OF AI VACCINATION

Biosecurity: a concept or a reality? All the history of controlling poultry diseases has taught us that vaccination without biosecurity is non sense. Some South East Asian countries have enormously improved their situation regarding the control of AI by closing their poultry houses. But in many countries, the economical and social situation explains the difficulties encountered to implement reliable biosecurity, and if efforts can be, and should be, made on this point, it is just not wise to exclusively promote or rely on this to control AI. In some cases, it is probably more efficacious to accept vaccination and make some investment on controlling the quality of the vaccines that are on the market and properly inform the users. Adaptation of prevention/control/ eradication programs to local realities is of paramount importance and no relevant advice can be given if local situation is not known and understood. Realistic objectives should then be determined.

Local authorities: strong help or roadblocks? There are countries where poultry industry is well organised and very much capable to implement proper control program. Coordination by local veterinary authorities can then reveal incredibly helpful as long as they are capable to understand the needs and constraints of the producers and work with them. Of course, this means that they are provided with sufficient degree of independence and freedom of action.

But there are also countries where the distance between authorities and poultry producers is so important that producers are just playing hide and seek with them. Then, the decision of vaccinating or not can be based on pure administrative speculations, and the "official" selection of a vaccine decided from criteria that are not exclusively technical…

The interest of following up infection and vaccination. Even if selected and applied, vaccination against AI cannot be considered as a permanently

acceptable solution. The virus is so contagious, so virulent, and possibly zoonotic that the final objective, even if on the long term, should be eradication. For these reasons, monitoring of both the circulation of the virus and the evolution and changes of its antigenic and biologic properties should be closely monitored, preferably inside the frame of an international collaboration. Serological methods to differentiate between infected and vaccinated birds (DIVA approach) have been described and should be considered as part of a sound vaccination plan. Complexity of it should take into account the human and money feasibility of it.

CONCLUSIONS

There is no perfect AI vaccine, no perfect AI vaccination, and there is no defined attitude regarding their use that could be applicable to all situations. Good quality AI vaccines are capable of bringing consistent clinical protection and help eradicating the disease by reducing the amount of challenge virus shed and duration of shedding in case of infection. Using it can be very beneficial in case of new epizootic getting out of control, and is almost unavoidable in some infected countries. Emphasis should be put on the importance of their quality as well as the importance of proper vaccination technique. Prevention program with vaccines should necessarily include action on the quality and reality of the vaccination as well as monitoring of vaccination and possible changes of the virus.

EFFICACY AGAINST VERY VIRULENT (VV) MAREK'S DISEASE VIRUS (MDV) OF THE HVT/ND RECOMBINANT (INNOVAX™- SB-ND) AND THE IMMUNITY AGAINST VIRULENT (V) NEWCASTLE DISEASE VIRUS (NDV)

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SUMMARY

The HVT/ND recombinant "Innovax-SB-ND" is a HVT vector with the F (fusion protein) gene insert of NDV in combination with the MDV vaccine SB1. The efficacy of MDV vaccines is officially based on challenge of *in ovo* or one-day-old subcutaneous vaccinated SPF chickens and challenged at five to six days of age with a virulent MDV.

Actual differences in protection between different types of commercially available MDV vaccines, including any HVT recombinants, can only be shown in MDV shedder challenge trials. The shedder trial mimics early exposure against vvMDV under field conditions. In a shedder trial, vaccinated groups and unvaccinated controls (contacts) are placed immediately after hatching in pens with chickens that were infected two weeks prior at one day of age with vv MDV (shedders).

To compare the efficacy of the HVT/ND recombinant and a conventional HVT vaccine, both in combination with SB1, a shedder trial was conducted using a vv + MDV as the challenge virus. No significant difference in protection was shown between the recombinant HVT/ND + SB1 and the conventional HVT + SB1 (data will be presented).

The HVT/ND recombinant does not induce any significant NDV hemagglutination inhibition (HI) antibodies and/or ELISA titers. Lack of serological response can be used as a marker ND vaccine in monitoring ND field exposure. Independent of the NDV maternal antibody level, excellent protection against vv NDV was obtained starting around two weeks of age and continuing, as far as tested, until 40 weeks of age after one day of age vaccination. (NDV challenge and serology data will be presented.)

In general no additional live NDV vaccines are required in chickens vaccinated with the HVT/ND recombinant vaccine which particularly in broilers prevents the problem of NDV respiratory vaccination reactions. Monovalent infectious bronchitis (IB) vaccines can be used, eliminating any interference that occurs using combined NDV/IBV vaccines.

COMPARISON OF DIAGNOSTIC TECHNIQUES FOR THE DETECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

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SUMMARY

Methods to diagnosis infectious laryngotracheitis (ILT) by 1) direct fluorescent antibody (FA) test on frozen tissue sections of trachea and conjunctiva, 2) histopathology on trachea and conjunctiva, 3) virus isolation in SPF eggs and confirmation by FA on frozen sections of chorioallantoic membrane, 4) virus isolation in cell culture and confirmation by electron microscopy, and 5) PCR have been compared.

Since October 2005 a strain of ILTV circulating in California has not been easy to propagate in embryonating chicken eggs. Results from this study showed diagnosis of ILT was more reliable when based on histopathology and FA findings on frozen sections of trachea and conjunctiva than by virus isolation, which has traditionally been considered the most sensitive test. These results correlated well with PCR testing of swab samples taken from the same cases.

FA testing and negative stain EM on CAMs from eggs inoculated with ILT-positive samples, as judged by positive FA testing on original tissues, were negative in many cases, indicating that the virus was not replicating in embryonating chicken eggs. In cases where FA testing on CAMs was positive virus was not always detected by negative stain EM. However, it is recognized that EM is less sensitive than other tests.

In our experience direct FA testing on frozen sections of trachea and conjunctiva offered a specific, inexpensive, reliable, and rapid diagnosis within hours of birds being necropsied.

FIRST RECORDED BREAK OF INFECTIOUS LARYNGOTRACHEITIS IN LOUISIANA

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CASE REPORT

The commercial poultry industry has been active in Louisiana for well over 20 years. In all that time, there has never been a recorded outbreak of infectious laryngotracheitis (ILT) anywhere in the state. This has not been due to lack of checking. Veterinarians working for the commercial poultry companies and for allied industries frequently check flocks for evidence of disease, and although a poultry diagnostic facility has not been located in Louisiana until recently, labs in surrounding states have been frequently used for diagnostics and disease monitoring.

In July 2006, a young commercial broiler breeder flock that had just recently been moved from their pullet farm in Arkansas began having higher than normal mortality with clinical signs of extreme respiratory distress with open mouth breathing and decreased egg production. Veterinarians were called to investigate the problem, and ILT was high on the suspicion list as the disease was currently in south Arkansas creeping its way close to the Louisiana border.

A veterinarian working with the company performed necropsy on several dead and sick birds, but at this time very few lesions were found. The lesions that were found were primarily very small opaque round lesions in the lungs, resembling aspergillous nodules. A few of the birds had caseous plugs found in the tracheal bifurcation. There was no obvious blood in the tracheas and no other evidence of what would be causing the seemingly extreme respiratory distress and death in these breeders.

The next day, the same veterinarian and the Louisiana Department of Agriculture and Forestry Poultry Diagnostic Laboratory director, also a poultry veterinarian, performed necropsy on more of the dead and sick birds. This time, several of the birds had yellow caseous material adhered to the larynx, focal spots in the upper trachea and esophagus, plugs in the bifurcation, and the small opaque nodules in the lungs.

The top rule outs at this point in time were fowlpox, ILT, trichomoniasis, and aspergillosis.

Tracheas, eyelids, and lungs were sent to Mississippi State University, University of Georgia Poultry Research & Diagnostic Lab, and The Georgia Poultry lab primarily to rule out ILT. All three labs returned results of ILT positive by various methods of histopathology, PCR, and IFA. The PCR further identified the ILT as being of vaccine origin. Lungs were also cultured at UGA and found to be positive for *Aspergillus fumigatus*. No evidence of fowlpox was found.

The farm had two houses, each divided in half by the egg collection/work room, effectively making a four house farm. The disease started in House 1, spread to House 2, then across to Houses 3 and 4. The farm had been quarantined by the company as soon as the clinical signs in House 1 were noticed. After the diagnosis, the farm was officially quarantined by the state Department of Agriculture & Forestry. It was decided not to vaccinate the flocks since the disease appeared to be self limiting after about 14 days with the highest mortality peaking at day six to seven at approximately 0.7%. Egg production returned to normal after about four to six weeks after clinical signs ended. Eggs were disinfected before being loaded and egg trucks were disinfected prior to leaving the farm. The closest farm to the break was about 1.5 miles and owned by the same company. No other breaks were ever found.

This unusual presentation of ILT could have easily been missed, but due to the diligence of the poultry company involved in calling in experienced poultry veterinarians and the willingness to find out what was going on, the diagnosis was made. After assessing the epidemiological parameters of the break, it was determined that the most likely cause of the break was introduction into the flock by the crew in Arkansas that loaded the pullets for transport to the hen houses in Louisiana. The unusual presentation of aspergillosis in the breeders is thought to have been initiated by the weakened immune system of the birds from the ILT in the face of contaminated rice hull litter.

MANAGEMENT METHODS TO REDUCE INFECTIOUS LARYNGOTRACHEITIS VIRUS

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Infectious laryngotracheitis virus (ILTV) is a contagious pathogen, which results in serious economic losses in commercial chickens worldwide. ILTV is controlled in pullets with individually applied highly attenuated vaccines. However, in areas of high concentration of chickens of all ages, ILTV is causing severe problems in broilers. Due to high labor costs, individual vaccination of broilers in the field against ILTV is not practical. Less attenuated vaccines must be administered in mass to broilers by drinking water or course spray, and these vaccines can cause severe reactions resulting in reduced performance. In addition, these vaccine viruses can survive in poultry houses, and after back passage (bird to bird transfer), can become more virulent causing a condition know as "silent LT or almond eye." This condition causes drops in broiler performance. Also, there is not sufficient vaccine produced in the US on a yearly basis to vaccinate all the broilers in affected areas. Therefore, management practices to reduce the ILTV concentrations in chicken houses are needed. We developed a natural challenge method, using sentinel chickens reared on litter contaminated with ILT back passed vaccine virus and a nested polymerase chain reaction (PCR) to determine the presence of ILT vaccine virus in the feces and tracheas of the chickens. Using these methods, we determined that several commercially available poultry litter treatments (Poultry Guard™, Al+Clear™, and PLT™), heating the litter to 38°C (100°F) for 24 hrs, and in-house composting for five days will inactivate ILT vaccine virus. This information is of immediate use to the poultry industry for controlling ILT vaccine virus induced disease in broilers and may reduce other important viral pathogens as well.

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A COMPARATIVE INFECTIOUS BURSAL DISEASE VACCINE FIELD TRIAL

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ABSTRACT

Recent investigations in a broiler ranch in California with a history of runting and stunting revealed the presence of an infectious bursal disease virus (IBDV) strain comparable to the designated TI strain isolated first in Georgia. Severe bursa damage detected via histopathology during this study and the detection of T1-like IBDV strain suggested a subclinical bursal infection in the ranch. Based on this previous investigation, three field trials were initiated to test the protective potential of five different commercial IBDV vaccines via histopathological examination.

Bursal damages were detected in all three studies caused by T1 strain. None of the used vaccines were effective to prevent the bursa lesions in the initiated trials.

WHAT HAPPENS TO THE GUMBOROVIRUS AFTER INOCULATION OF MATERNAL ANTIBODY POSITIVE BIRDS? WHERE DOES IT SURVIVE?

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SUMMARY

Field observations and laboratory studies indicate that the outcome of Gumborovirus (infectious bursal disease virus, IBDV) vaccination may vary depending on the maternal antibody level at the time of vaccination and the genetic background of the birds (1, 2). It is not known where in the infected chicken IBDV may last in the presence of maternally derived antibodies (mAb) before the antibodies levels are low enough for the virus to start replicating (4).

In this study we compared the pathogenesis of an intermediate IBDV vaccine strain in specific-pathogen-free (SPF) layers and commercial broilers with residual mAb. Birds were inoculated either *in ovo* or orally post hatch. Groups of embryos and 14-days old chickens of the different genetic backgrounds were inoculated with 10^4 tissue culture infectious $dose_{50}$ of an intermediate IBDV strain. The same number of embryos and birds was inoculated with virus-free diluent. At 4, 6, 10, 14, 21, 28, and 35 days post inoculation (PI), five birds per group were sacrificed, serum samples collected, histological bursa lesions determined, and IBDV distribution in different tissues investigated.

The detection of IBDV-serum antibodies by virus-neutralization test revealed that at the time of vaccination broiler embryos and broiler chickens still had had significant mAb levels. Seroconversion was observed by ELISA at 10 days PI in 100% of the IBDV-inoculated SPF-birds and in 24% of the post-hatch inoculated broilers. The first IBDV-antibody positive birds in the *in ovo*-inoculated broiler group were detected at 28 days PI. The severest bursa lesions were observed in the *in ovo*-inoculated SPF-layer type chickens, which peaked between 4 and 6 days PI. The peak of bursa lesions in the post hatch inoculated SPF-layer type chickens was observed between 10 and 14 days PI. Post hatch inoculated and *in ovo* inoculated

broilers showed mild bursa lesions beginning at 14 days and 35 days PI, respectively. These observations indicate that despite the presence of different levels of antibodies, IBDV infection occurred both in post hatch and in *in ovo*-inoculated broilers.

To determine the presence of IBDV in spleen and bursa cloacalis, we used three detection methods: immunohistochemical IBDV-antigen detection, virus-isolation (only for the post hatch inoculated groups), and detection by quantitative RT-PCR. Significant differences between infected groups were found not only in the timing but also in the tissue type from which replicating IBDV was isolated. In the post hatch inoculated SPF-layer-type birds IBDV-antigen was detected by immunohistochemistry in the bursa cloacalis between 4 and 14 days PI, and in the *in ovo*-inoculated SPF-layer group between 4 and 10 days PI. In post hatch inoculated broilers, IBDV-antigen was detected in the bursa cloacalis only in two of 10 birds between 21 and 28 days PI, while none of the *in ovo*-inoculated broilers showed IBDV-antigen in any tissue. No IBDV-antigen was detected in spleen tissue at the investigated time points.

Replicating IBDV was re-isolated *in vitro* from bursa homogenate and spleen. Samples were collected between 4 and 21 days PI from post hatch-inoculated SPF-layer as well as broiler type chicken (Table 1). The bursa samples from inoculated broilers taken between 4 and 10 days PI were positive for replicating virus although no bursa lesions or IBDV antigen were detected at these time points. Detection of IBDV by quantitative RT-PCR revealed that the amount of IBDV in bursa and spleen varied between groups (Figure 1). Not only the presence of maternally derived antibodies and the genetic background, but also age may have a significant influence on IBDV-replication. While large amounts of IBDV were found in the bursa cloacalis of post hatch inoculated SPF-layers, *in ovo* inoculated birds had higher quantities of IBDV in the spleen (Figure 1). The post hatch inoculated broilers were positive for IBDV by RT-PCR only on day 28 PI (data not shown).

In order to identify, what types of cells were IBDV-positive after inoculation of antibody-positive birds, SPF-layer type chickens and broilers with mAb were inoculated in a second experiment with an intermediate IBDV strain. At 7 and 14 days PI, birds were sacrificed and spleen and bursal tissue investigated for IBDV by RT-PCR. Adherent and non-adherent, as well as B cells and macrophage-type cells of these tissues were isolated and tested for IBDV. At the investigated time points no IBDV was detected in spleens of SPF-layer type birds. Different to the SPF-layer type birds, in infected broilers the non-adherent spleen cells were IBDV-positive. Interestingly, SPF-layer type birds showed IBDV-positive adherent macrophage-like cells in the bursa cloacalis at 7 and 14 days PI. In infected broilers non-adherent bursa cells were IBDV-positive, which were further identified as B cells after cell separation with magnetic beads.

Our study not only shows that maternally derived antibodies influenced the onset of IBDV infection and delayed it in comparison to antibody free birds, we also demonstrated that the target cell populations may differ between SPF-layer type birds and maternal antibody positive broilers. If these differences are only due to the influence of maternally derived antibodies, or if the genetic background may also play a significant role, is not clear. Previous studies by our group have indicated that the genetic variation may be neglected with respect to the pathogenesis of an intermediate IBDV strain (3).

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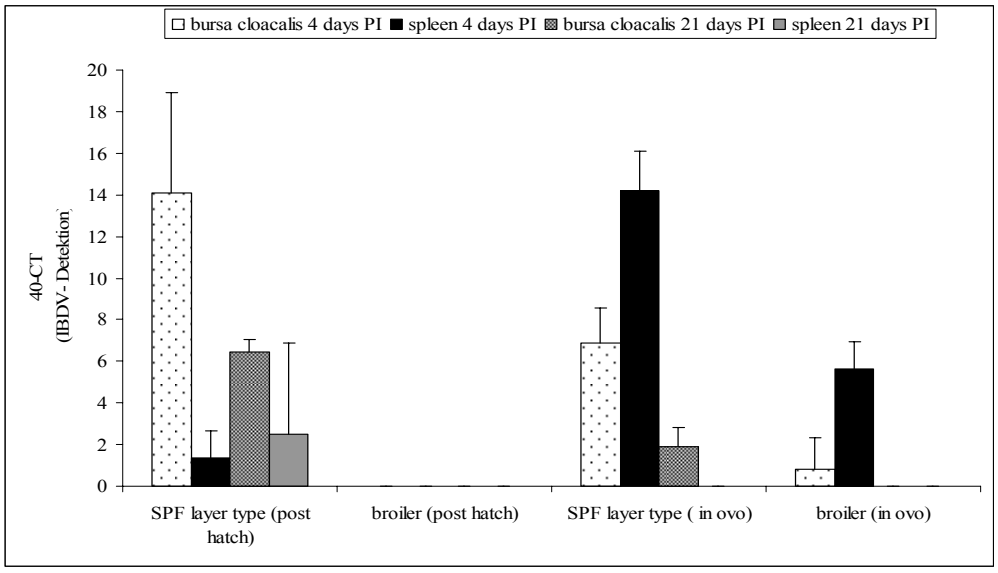
Table 1. IBDV isolation from bursa cloacalis and spleen of IBDV-infected broilers and SPF-layer-type chickens.

IBDV-inoculated groups	Tissue samples were collected at days PI									
	4		6		10		14		21	
	bursa	spleen	bursa	spleen	bursa	spleen	bursa	spleen	bursa	spleen
SPF-layer-type	+	+	+	-	+[2]	-	+	-	-	-
Broiler	+[2]	+	+[2]	+[2]	+[2]	+[2]	-	+[2]	-	-

+ = isolation of replicating IBDV on chicken-embryo-fibroblasts; - = no detection of IBDV after 3 passages on chicken-embryo-fibroblasts. [2]detection of replicating IBDV after the 2. passage; all the other samples were IBDV-positive with cytopathic effect in the first passage. Samples of virus-free birds were IBDV-negative. From each group, 5 individual samples of the same tissue were pooled and homogenized.

Figure 1. Detection of IBDV by quantitative real-time RT-PCR in individual tissue samples of IBDV-infected birds. Tissue samples of virus-free birds were IBDV negative. At 14 days post hatch or at 18 days of embryonation, birds were inoculated orally with IBDV. CT = corrected cycle threshold; n = 5.



FIELD USE OF FEATHER PCR DIAGNOSTIC TESTING TO OPTIMIZE MAREK'S DISEASE VACCINATION

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ABSTRACT

Vaccines have been used since the early 1970s to control Marek's disease, an oncogenic and immunosuppressive disease that causes economic losses in chicken production. With field virus and vaccine pressures, this virus over time has increased in virulence requiring continual monitoring and evaluation of current control methods.

It has been found that feather follicular epithelium and feather tips are sites of productive Marek's disease viral replication (6, 7, 8). Using quantitative polymerase chain reaction (PCR), virus load in feather tips is indicative of virus load in the spleen, which is the predominant site of immune responses to Marek's disease viral antigens (5, 9). A real-time PCR method has been developed and validated to quantify Marek's disease virus genomes in feather tips (4). Various tissues were evaluated and feathers were found to be the tissue of choice for sampling for PCR confirmation of successful vaccination of commercial chickens (3). Determination of a feather Marek's disease viral genome profile is a convenient way to measure the level of vaccine multiplication and show a flock's response to vaccination (1, 2).

Knowing that various factors, such as bird breed, can affect vaccination effectiveness, this diagnostic approach using PCR aids in the determination of the optimum Marek's disease vaccination program best suited for an individual production system. Field use of this technology includes vaccination monitoring, vaccination program validation, selection of genetic breed, determination of outbreaks, and field surveys.

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INTERESTING FIELD CASES (EXPERIENCES) INVOLVING COMMERCIAL POULTRY

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INTRODUCTION

My plan is to share with you several field cases that I found interesting over the past several years. These are not case studies but actual disease situations involving small commercial poultry operations with normal management practices, which sometimes do not allow quick, easy solutions to resolve the problem.

CASE 1
PIGEON PARAMYXOVIRUS SEROTYPE 1 (PPMV-1) IN SQUAB PRODUCTION

Squab are specially bred nestling pigeons four to five weeks of age raised for meat production for the "High End" restaurant trade in the U.S. and Canada. Market weights range from 1.2 to 1.5 pounds. Squab are raised and fed by their parents in nests made of bird droppings and pine needles. Production units have multi-aged breeders and various aged young throughout the year. The size of a typical squab production unit will vary from 200 to 5,000 breeding pairs of pigeons. Breeder candidates are selected by the producer several times a year or are purchased in small numbers from other producers. A good breeding pair will raise around 16 to 20 plus young in a calendar year.

Pigeon paramyxovirus 1 (PPMV-1), a variant of Newcastle disease, is a highly infectious and acute disease of pigeons caused by avian paramyxovirus serotype 1. The virus infects the membranes of the pigeon's intestinal and respiratory tracts as well as the nervous system. Clinical signs include diarrhea, weight loss, ataxia, head tremors, and torticollis. Mortality in adults is around 10 to 30%; however, mortality in young squab can be 30 to 80%. Every year several squab growers in Central California have to deal with this disease.

What makes PPMV-1 so devastating for squab producers? Unlike your traditional poultry operations that get infected with the local endemic PMV-1 strains (Newcastle disease) in which the course of the disease may run for several weeks, PPMV-1 in a squab operation may last up to three to four months. The economic impact of PPMV-1 may last longer – up to six to seven months in a squab facility. This was brought to my attention in 2000 - 2001 when we had over eight PPMV-1 cases in the greater Central California area. Clinical signs would appear in several lofts in a building then slowly move through the entire production facility over several months. The lofts that were the first to show clinical signs would recover in two to three weeks and the entire operation in about three to four months. Serology results also back up the assumption that the PPMV-1 virus moves slowly through a squab production facility.

Squab producers are fairly independent-minded individuals that draw from years of experience and sometimes-antiquated knowledge on how best to deal with this disease. Needless to say it has been an uphill battle to educate the growers on the importance of biosecurity and the use of a killed vaccine. Much headway was made when it was pointed out that those growers who had been affected by PPMV-1 took almost four to six months to return to their normal shipping numbers of squab, thus affecting their financial well being. This was primarily due to the following:

• *10 – 30% adult breeder mortality*. This forced the producer to find the single parent and rearrange new breeding pairs. Otherwise no production would take place in the nest. If a producer wanted to purchase additional adult breeders, one would have to contact other producers to see what the availability of adult and/or breeder candidates were. The producer would have to save additional squab as replacement breeders to replace the adult breeders that died.

• *30 – 80% young squab mortality over the next several months*. Most of this mortality is most likely due to parents who are affected by the virus and for whatever reason are unable to feed or care for their young. Reproductive behavior and egg production would visibly decrease for several weeks after the birds appeared to recover from the infection.

The financial loss of market-aged squab to the producer ranged from $3,000 to 11,000 plus. In addition, the processing plant lost product and sales to their competitor as well as having a negative impact on the plants reputation as a supplier.

The squab growers have been invited to a number of educational meetings where biosecurity procedures and protocols have been extensively reviewed. These protocols included reducing feral pigeon exposure; designated clothing and footwear for the farm and when shipping birds to the processing plant; testing birds before purchasing; pest control; adding gates; and reducing visitors to the farm. In addition, PPMV-1 vaccination programs (Inactivac PMV-1 Main Biological Laboratories) have been implemented for those growers with previous exposures or if located in an area where PPMV-1 is prevalent. Breeders are vaccinated twice, four to six weeks apart, and again annually. Since the implementation of this program, growers who have routinely vaccinated for PPMV-1 have not been diagnosed with the disease. The vaccination program unfortunately has not been thoroughly accepted by most of the growers primarily because the effort to catch and handle each bird to be injected as well as coordinating the vaccination program with the periodic addition of breeder candidates.

PPMV-1 vaccine has also been used in the face of an outbreak. Two farms, which showed clinical signs of PPMV-1 and confirmed with lab diagnostics, immediately vaccinated the entire flock. Pigeons located in buildings farthest from the initial clinical signs were first vaccinated. Farm "A" vaccinated all 4,000 adult breeders in approximately 18 hours. Clinical signs including mortality were held to 4 of 14 buildings with minimal impact to the grower. Farm "B" vaccinated approximately 5,000 breeders in about a week. Clinical signs including mortality were held to 3 of 21 buildings with minimal impact to the grower. Although this practice of waiting to vaccinate a flock until clinical signs are observed is not encouraged for several reasons, it has given hope to many producers that they can reduce the economic impact of PPMV-1 on their squab production through use of the PPMV-1 vaccine upon exposure.

CASE 2
LOW LPAI (H6N2) IN A QUAIL BREEDER/COMMERCIAL OPERATION

In July of 2004 a small quail breeder / commercial operation located in the Central Valley of California submitted several three-week-old commercial quail with swollen hocks to the California Animal Health Food Safety (CAHFS) Lab in Turlock, California for diagnostic services. The quail were diagnosed with fowl cholera caused by *Pasteurella multocida*. In addition, an avian influenza virus (H6N2) was isolated from the submitted quail. The significance of this outbreak is that the quail facility is located near two large layer operations as well as a nearby commercial poultry feed mill, and that it had been approximately a year since the last confirmed AI (H6N2) outbreak on a commercial poultry farm in Northern California. The concern here was that this was a new AI outbreak and how will the owner eliminate the virus from the flock/premise and still stay in business.

The quail farm consisted of one building with approximately 450 breeders of various ages and one building with 9,000 commercial quail ranging in age from one to nine weeks of age. In addition, the owner purchased brown pullets and had them delivered to the quail farm on a weekly basis. The brown pullets stayed on the farm for one day before marketing the pullets at a nearby processing plant along with the commercial quail.

The owner was very cooperative in wanting to make every effort to eliminate the virus from his facility. He was concerned that the government and/or poultry industry representatives would push for the depopulation of his breeders as a way to mitigate the problem due to the immediate location of the operation to several major layer operations.

The following protocols and procedures were reviewed and discussed with the owner. The owner implemented an immediate "Self Quarantine." No outside birds were to be brought to the farm (brown pullets). All eggs in the incubator were to be destroyed. No new placements of young quail were to be hatched and placed on the farm for a minimum of eight weeks. The commercial quail would be controlled marketed through the processing plant. All manure would be removed and covered onsite. The facility would then be cleaned and disinfected.

Blood and swab samples were taken to assess the influenza situation currently on the farm. The breeders (10 birds) were virus negative but serologically positive for AI (H6N2 and H1N2). Eight-week-old commercial quail were serologically positive (8/9 birds) and virus negative (25 birds). It was determined that with each new weekly placement of commercial chicks, the virus stayed "active" in the young three-week-old quail, allowing the virus to remain on this facility for an extensive period of time. The thought here was that this flock was probably positive for AI when the nearby layer facilities were positive with H6N2 avian influenza a year earlier and remained undetected until the birds exhibit clinical signs of a bacterial infection and birds were submitted to the lab for further diagnostics.

The processing plant and owner requested a plan to move hatching eggs offsite where the eggs could be incubated, hatched, and commercial quail grown for market so that their would be minimal disruption in

growing and processing quail. This will be discussed during the presentation.

In October of 2004 ten six-week-old commercial quail from the breeder ranch were submitted to the lab to determine the farm's AI status post cleanup. Because these birds were hatched from the original breeders onsite and raised in the adjacent building, the submitted birds acted as sentinels. Virus isolation and serology were negative for AI.

CASE 3
RUNTING AND STUNTING IN COMMERCIAL BROILERS?

During the latter part of 2003 a small commercial broiler company was having issues with uniformity both in the field and in the processing plant. Market age broilers were two to three days behind in body weight, but as several months went by, we were looking at seven days-plus delay in some instances. In addition, the broilers exhibited feathering problems, gait abnormalities, and poor uniformity issues.

During the initial investigation birds from two to four weeks in age were submitted to the lab from several farms in an attempt to find the cause of the "Runting and Stunting" problem. Mild coccidiosis and necrotic enteritis was the initial diagnosis. But this was commonly seen throughout the company prior to this time period. In addition, a commercial cocci vaccine was being administered to all commercial birds for cocci control. There were no production issues observed on the farms that we were able to determine that would lead to the problem at hand. As the runting and stunting issues continued to increase we were able to narrow the incidence down to only those broilers grown as Organic. This was made apparent where the Organic and Antibiotic Free (ABF) broilers were raised on the same farm but in different buildings. The ABF broilers were marketing as scheduled but the Organic raised broilers were showing poor feathering, weight gains, and size variations typical of Runting and Stunting.

Several feed samples from both the Organic and ABF diets were submitted for analysis. Nutrient specifications were in line with the nutritionist's expectations. No mycotoxins were detected. Diagnostic results from submitted birds ruled out viral enteritis, reovirus, bacterial septicemia, and rickets. Only the occasional bird with necrotic enteritis or mild coccidial enteritis was observed in both ABF and Organic grown broilers.

The above information was reviewed with the consulting nutritionist several times. Basically, our attempt was to try and rule out those possible disease agents and management issues that could lead to Runting and Stunting. The question became clearer as time went by that there was something different regarding the feed ingredients of the Organic feed versus the ABF feed that was causing this growth problem seen in the field.

After several attempts to persuade the consulting nutritionist to take a second look at the raw feed ingredients, it was determined that the organic soybean meal being used for the organic broiler production over the past several months was undercooked. Raw or undercooked soybean meal contains trypsin inhibitors. The proteolytic-inhibiting effect of the trypsin inhibitor reduces the growth rate of chickens. What was interesting about this case was the length of time and effort it took to narrow down the possibilities and get a final diagnosis. Getting the final diagnosis was a group effort of several poultry professionals working together.

CASE 4
SWINE INFLUENZA IN TURKEY BREEDERS (BRITISH COLUMBIA, CANADA)

History of swine influenza in the Fraser Valley. The Fraser Valley of British Columbia (BC) is a rural agricultural area containing an abundance of closely interspersed mixed livestock commodities including swine and poultry. Most of the commercial swine industry in BC is served by a highly competent swine practitioner who became aware of the introduction of swine influenza onto BC pig farms in the fall of 2004. Since the progress of the outbreak in pigs was slow and the clinical signs minor with a high rate of recovery, the swine practitioner promoted extensive use of prophylactic vaccination throughout the swine industry with generally successful results. Due to the proficiency of this practitioner and the lack of regulatory reporting requirements for swine influenza, the BC Ministry of Agriculture & Lands-Animal Health Centre was unaware of the outbreak occurring in swine until in May 2005 a local swine producer submitted six-week-old pigs to the Animal Health Centre (AHC) for diagnostic investigation of coughing and anorexia. Nasal swabs were positive for swine influenza (H3N2) by PCR and egg inoculation, although no virus was recovered by inoculation onto CKC, CEFC, and MDCK cells.

BC has only one turkey breeder operation situated in the Fraser Valley amidst densely populated commercial swine (~30) and poultry (~600) farming operations. This turkey breeder farm was directly across the road from one of the commercial swine operations that was experiencing an active outbreak of swine influenza. In May of 2005 a 38-week-old commercial Hybrid turkey breeder flock (flock A)

housed in four buildings suddenly experienced a drop in egg production and an increase in white-shelled eggs. Breeders were vaccinated for hemorrhagic enteritis, pox, avian encephalomyelitis, cholera, Newcastle, and paramyxovirus serotype 3 (except the toms). Initial clinical signs were that the flock appeared clinically normal except for an increase in white-shelled eggs 3 to 15% combined with a 5 to 10% drop in egg production on a daily basis. The mortality was virtually unchanged. In fact, the ranch manager felt mortality actually was improving as egg production was dropping. Breeders housed in buildings 3 and 5 were first to exhibit the decrease egg production followed by buildings 2 and 4 three to seven days later. Within 10 days after the initial egg production drop, hens were laying approximately 8 to 12% egg production. Because of the sudden decrease in egg production and the increase in white-shelled eggs, paramyxovirus serotype 3 (PMV-3) was suspected. Blood from both the hens and toms were submitted and tested for PMV-3. Toms had 2/10 samples positive for PMV-3. Hens had negative to moderate titers to PMV-3. Because of the low PMV-3 titers and the fact the toms had two samples positive for PMV-3, additional blood and birds were submitted to the AHC for further diagnostics – specifically to rule out PMV-3 and influenza.

Lab results were as follows: PMV-3 results were unchanged; serology and PCR were positive for influenza; no virus was initially isolated. The influenza was typed as H3N2 (Swine Flu). The birds were in excellent body condition with abundant muscle mass and fat reserves. Hydration was adequate. The respiratory tracts were clear. All birds were in full egg production with eggs present in the shell gland. There was marked congestion of the ovary associated with a milky effusion. There was mild fibrinous airsacculitis and the abdomen contained a mild increase in fluid that contained flocculent fibrin. There was mild splenomegaly and an occasional bird had patchy pulmonary congestion.

The routine in-lab screening test for influenza A in most avian species is matrix RT-PCR on tracheal swabs or affected respiratory tissue (sinus, lung, trachea, pleura). In this case, in the majority of the swine influenza-infected turkeys the samples taken from the trachea, cloaca, liver, lung, kidney, air sacs, cecal tonsil, spleen, and brain were all negative for influenza A by PCR and virus isolation. In contrast, the same birds were strongly positive for influenza A by PCR on the ovary and oviduct, suggesting a strong ovarian predilection for this virus. The significance of this finding suggests that routine screening methods for influenza A using tracheal and/or cloacal swabs will not be sufficient to detect swine influenza infection in turkey breeders. This may also explain why this virus does not easily infect sexually immature meat turkeys.

Despite the detection of swine influenza (H3N2) virus by PCR it was extremely difficult to isolate this virus from both swine and turkey tissues. Duplicate samples were forwarded to the National Centre for Foreign Animal Disease in Winnipeg and through careful and meticulous manipulation Dr. John Pasick was successful in recovering the virus from eggs. The viral isolate from the BC turkey breeders (A/TU/BC/05) has the closest H3 sequence homology with similar isolates from turkey breeders from the Midwest in previous years (A/TU/MN/03 and A/TU/NC/04). The N2 sequence is most closely related to A/Human/NY/03.

The farm was immediately self-quarantined. The swine farm located across the street was notified of the turkey farm's situation only to find out that the swine farm had been dealing with swine influenza for the past several weeks. Flock A was depopulated five weeks later when it was apparent the breeder flock would not come back into production.

An attempt was made to develop an autogenous swine influenza vaccine for the commercial turkey breeders. After several months of negotiations with CFIA officials, a U.S.-based vaccine company was unable to procure the original virus. Steps then were taken to import a killed influenza vaccine H3N4 to be used to vaccinate flock B that was approximately 14 weeks of age. Flock B was located at the brood/grow farm at a different location. Because of delays in the permit and import process, flock B was only able to receive two injections of the vaccine versus the schedule three applications. Prior to moving to the lay facility in early September 2005, flock B (28 weeks of age) tested positive for influenza (22 / 30), which was expected.

In late October flock B experienced a sudden drop in egg production and an increase in white-shelled eggs. Blood and birds were submitted to the lab. The flock was positive on PCR for H3N2 swine influenza. Flock B was depopulated. Discussions then took place with the owners as to how we were going to handle flock C, which was 10 weeks of age at the time located at the brood/grow facility.

An attempt was made to expose flock C to the swine influenza virus. Twenty, 10-week old breeder candidates were bled and then transported to the lay facility. Five birds were placed in each of the four lay barns for 7 to 10 days and then moved back to the brood/grow facility. At the same time 10 hens from the lay facility, five hens from a barn in the initial stages of an egg production drop, and five hens from a barn as yet to show clinical evidence of an egg production drop were transported to the brood/grow facility. Flock C

showed no serological evidence of exposure. Flock C was then vaccinated twice with the killed H3N4 influenza vaccine.

In addition to the failed attempt to exposed Flock C to the H3N4 virus, a decision was made to "swap" the lay and brood/grow facilities. The theory was to brood/grow and darken the breeder candidates at the original lay facility located adjacent to the swine farm. This was an effort to get the breeder candidates exposed to the virus prior to production or to (hopefully) move clean unexposed breeder candidates to the old brood/grow facility where isolation/biosecurity would hopefully protect future lay flocks. Flock C was raised entirely at the original brood/grow farm and then put into production on the same farm (brood/grow). Flock C went through its entire production cycle with no egg production issues.

Flock D was brooded at the old lay facility adjacent to the swine farm. Flock was serologically negative for influenza at 20 weeks of age. Flock D was moved to the new lay (old brood/grow) facility a 29 weeks of age. Routine serology at 35 weeks of age revealed that flock D was positive for influenza (24 of 50 samples on AGID). No clinical signs were evident in the flock. House 4 peaked at 69% and house 2 peaked 60% egg production. Additional blood was submitted at 38 weeks of age and found 5 of 49 blood samples positive for influenza. Hens recently out of production were submitted to the lab in an attempt to recover a virus. Submitted birds were PCR negative for influenza. During the writing of this paper attempts were being made to serotype the influenza. Because no blood samples were taken just prior to moving the flock to the lay facility, we can only assume that flock D was exposed to the influenza virus sometime after 20 weeks age during the darkening period and prior to moving to the new lay farm (old brood/grow). This assumption is based on lack of clinical signs of influenza in flock D.

Further discussions from the farm management revealed that one house of breeder candidates started to lay eggs at 23 weeks of age due to a lighting issue. The flock was subsequently forced out of production. It is theorized that having functional ovaries for a week or so allowed the breeder candidates to become susceptible to the influenza virus. Discussions with the neighboring swine farm did not reveal any swine influenza issues. To my knowledge, none of the commercial turkeys are currently showing any seroconversion to or evidence of infection with the swine influenza virus.

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CONTINUING EDUCATIONAL NEEDS OF POULTRY PRODUCERS, 1994-2006

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ABSTRACT

The threat of H5N1 avian influenza entering into North America has driven the need to provide continuing education to poultry flock owners. Because of this concern, there has been an expansion of avian health resources provided to the poultry flock producer. These resources have ranged from written resources to oral presentations. This 12-year study examines the types of continuing education topics requested by poultry flock owners and determines if educational needs have shifted based on the threat of global diseases. Over the twelve-year study period, questions were obtained from flock owners and categorized based on the type of disease concern in order to predict the trends in educational needs for poultry producers. For each year of the twelve year study, disease topics were categorized into digestive, respiratory, musculoskeletal, reproductive, neurological, nutritional, and management. A chi-squared test was used to determine if there were significant differences among the categories of health topics requested for each year. The results indicated that there were no significant fluctuations in the categories of topics requested and that there are specific diseases that poultry producers indicate the need for continuing education. These results will provide useful information to those providing outreach activities to poultry flock owners and maximize continuing educational training resources, especially in light of global disease threats.

(A full-length article is being submitted for publication in the *Journal of Extension*.)

VISCERAL GOUT CAUSED BY RENAL DYSPLASIA IN GROWING NINE-WEEK-OLD LEGHORN EGG STRAIN PULLETS IN NUEVO LEÓN, MEXICO

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SUMMARY

A flock of 30,000 growing Leghorn egg-strain pullets 11-weeks-old in Montemorelos Nuevo León, México started three weeks ago with high mortality. Severe lesions were observed in kidneys and ureters, which presented visceral gout. Other affected organs were heart, liver, and spleen. Samples were submitted for histopathology. Feed analysis showed an increase of 1.84 for protein, 2.96 for ash, 1.66 for calcium; only phosphorus was normal. Enrofloxacin was used and mortality was reduced. However, the withdrawal of enrofloxacin increased the mortality again. Bronchitis revaccination (Mass strain) was without results.

Microscopically, it was observed renal dysplasia which seemed to be the cause of renal failure, resulting in enhancement of urate deposition and mineralization. The extra high protein and calcium on the diet along with dehydration could have exacerbated the condition. It was concluded in this study that renal dysplasia was an important cause of visceral gout and should be taken into consideration as genetic factor as an etiology of visceral gout.

INTRODUCTION

It is accepted that majority of Leghorn laying hens had change during the last 5 to 10 years; therefore, nutritional management has become critical. Today, age maturity has been reduced almost a day per year.

Kidney dysfunction often leads to either visceral or articular gout, and/or urolithiasis. Visceral gout is a condition that has been recognized for more than 35 years. The increased substrate load to the kidney eventually leads to precipitation of calcium monosodium urates crystals. This is characterized by white chalk-like deposits either in synovial fluid and covering tendon sheaths of various joints, or on the serous surface of various abdominal organs, such as well as the heart sac.

There seems to be a genetic basis for gout, although, interestingly, the articular and visceral forms rarely are seen together. Urolithiasis and gout together are responsible for significant losses in poultry. In each situation, there is kidney dysfunction, and frequently most birds die from kidney failure. In this condition, the kidney function has decreased to the point where uric acid accumulates in the blood and body fluids. The uric acid subsequently precipitates as calcium sodium urate crystals in the kidneys and onto the serous membranes of the liver, heart, air sacs, and joints. The damaged kidneys are characterized by atrophied or missing portions of kidney lobes, urolithiasis, and tophi in remaining kidney parenchyma.

In many situations, kidney damage is precipitated or aggravated by viral infection like infectious bronchitis and avian nephritis virus. Certain viral strains of bronchitis, termed nephropathogenic, have a special predilection for kidneys.

The cause of gout is often difficult to determine. The original kidney damage may occur long before the onset of gout mortality. Possible factors that can cause or contribute to gout are nutritional, infectious, toxic, and others (genetic). If a viral infection is not a factor in the occurrence of kidney problems, then attention is usually focused on diet constituents – particularly, protein, minerals, and water supply.

This report suggests that there is a decrease in the fractional tubular resorption of calcium altered by genetic cause.

CASE REPORT

A poultry facility with a flock of 30,000 growing Leghorn egg-strain pullets 11 weeks old in Montemorelos, Nuevo León, México, with a history of long production for many years, was divided in two open houses of basic design. At the beginning of the onset (nine weeks old) management was done as usual and care and vaccination was according with the farm calendar.

Problems began with daily mortality from nine weeks old and gradually increasing until week 11 when we received samples in our lab. The accompanying clinical story described severe lesions on kidney, and the ureter were filled with urates (visceral gout) in kidneys, heart, liver, and spleen. Gross examination revealed white chalky deposits in kidney, and the surface as heart sac, liver, and other abdominal organs (mesenteries, air sacs, and spleen) and muscle surface. Feed analysis showed an increase of 1.84 for protein level, 2.96 for ash, 1.66 for calcium; only phosphorus was at normal levels. Mortality decreased when enrofloxacin was used; however, when withdrawn, mortality increased again. Bronchitis revaccination (Mass strain) was without results, but diagnosed as visceral gout or bronchitis nephrotrophic strain.

Organ samples included submitted for histopathology analysis were kidneys, ureter, spleen, liver, lung, trachea, ceca, heart, and ovary from nine-week-old pullets and were fixed in 10% formalin.

Histopathology. Microscopically there were different grades of maturity in the renal tissues, some glomeruli appeared compact and small while others were hypertrophied. Renal tubules appeared tortuous with hypercellularity of epithelium. There was discrete fibroplasia in the interstitium with zones of myxomatous appearance. Other changes included accumulation of mucoid material with uric acid crystals and partially mineralize detritus in convoluted tubules. Also, there was interstitial lymphocyte and macrophagic infiltration. In some cases, the response due to the presence of uric acid induced necrosis, epithelioid macrophage and heterophil infiltration. Furthermore, thrombosis and vasculitis in small blood vessels of medulla were observed. Ureters showed obliteration of the lumen due to accumulation of mucinous exudate mixed with calcified materials and heterophils. Morphologic diagnosis included: renal dysplasia, lymphocytic and granulomatous nephritis associated to urate crystals and calcified material, alone with suppurative ureteritis with luminal obliteration. Furthermore, suppurative pericarditis with zones of urate deposition and mineralization were present.

CONCLUSION

It is concluded that this type of problem can have more than one cause, but the genetic factor must be considered – especially with avian commercial laying hen lines. Lines must be selected that do not express this problem or have a minor expression of it. Urine acidification can be a helpful treatment but considering

not to induce a generalized metabolic acidosis. From the nutritional viewpoint, the renal dysfunction can be minimized by taking care that you do not oversupply nutrients such as calcium, crude protein, and electrolytes in the diet.

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Table1. Mortality by week.

Week	Dead birds
1	54
2	112
3	19
4	79
5	27
6	34
7	56
8	138
9	286
10	455
11	430

UTILIZATION OF INFRARED THERMOGRAPHY IN BROILER BROOD MANAGEMENT – CASE REPORTS

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SUMMARY

The importance of optimum broiler brood management in achieving genetic potential is becoming increasingly significant. In order for broiler chickens to achieve genetic potential in terms of, for example feed efficiency and body weight gain, optimum brood conditions are considered essential.

The modern broiler reaches market weight in fewer days and in turn spends a significant percentage of its lifetime in the brood chamber. Optimum brood husbandry is considered an extremely important component in any broiler production operation. In the field broiler brood conditions have been presented which in turn result in poor performance and perhaps disease.

Poultry service persons, including poultry veterinarians, are often presented with cases where less than optimal flock performance is experienced in one barn or in all barns owned and operated by a single producer. An evaluation of brood management is considered an extremely important exercise and often results in the discovery of a management deficiency that can help explain the reasons for poor performance.

Currently an infrared thermometer is a common tool in the evaluation of temperatures in the brood chamber and in helping to identify the zone of comfort where the chicks will be most likely to access feed and water. Recently the use of infrared thermography has proven extremely valuable in accurately evaluating the entire brood chamber and as a visual educational tool for producers and poultry service persons.

Thermography is the use of an infrared imaging and measurement camera to "see" and "measure" thermal energy emitting from an object. Thermal, or infrared energy, is light that is not visible because its wavelength is too long to be detected by the human eye.

The utilization of infrared thermography in large animal veterinary medicine is common (1). Recently, the utilization of the infrared camera in evaluating poultry barn ventilation systems and in poultry research has received significant attention (2, 3, 4).

As the poultry industry strives to improve performance, manage energy, improve food safety and quality, improve animal welfare, enhance worker safety, and reduce environmental impact new applications of infrared cameras will continue to emerge.

The utilization of infrared thermography in broiler brood management is described in specific field cases where poor performance, in terms of poor feed conversion, was noted as the producer complaint.

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DIFFERENT STRAINS OF *CAMPYLOBACTER JEJUNI* COMPETE FOR COLONIZATION IN BROILER CHICKS

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ABSTRACT

Campylobacter jejuni isolates possess multiple adhesive proteins (adhesins), which promote the organism's attachment to intestinal and cecal epithelial cells. In this study we selected two genetically distinct *C. jejuni* strains and generated one nalidixic acid resistant strain and a second streptomycin resistant strain. Both *C. jejuni* strains were motile and expressed known and putative adhesions as judged by real-time RT-PCR. The streptomycin resistant strain of *C. jejuni* was found to significantly reduce the efficiency of intestinal and cecal colonization by nalidixic acid resistant strain of *C. jejuni* in broiler chicks. This study revealed that *C. jejuni* strains could compete with one another for the colonization in chicks. This suggests that it may be possible to design novel intervention strategies to reduce the level at which *C. jejuni* colonizes.

INTERACTION OF IONOPHORE AND VITAMIN E IN THE KNOCKDOWN SYNDROME

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ABSTRACT

Monensin is a carboxylic ionophore commonly used as an anticoccidial drug in commercial poultry feed. Monensin has been associated with skeletal myopathy and cardiomyopathy in turkeys. Most of the cases reported have occurred in turkeys receiving doses of monensin in excess of the approved usage level or in combination with potentiating antibiotics (1, 2, 3). However, skeletal myopathy compatible with ionophore toxicosis also has been described in turkey flocks where monensin concentration in feed was within therapeutic levels (4).

Until recently, the primary diagnostic specimen to assess monensin concentrations was feed. However due to the uneven mixing of monensin, several feed samples from different sites have to be analyzed to give an accurate measure of its concentration. Another problem exists when the submitter does not provide feed samples. Diagnosis based on clinical signs and histology is not definitive, as these changes are not exclusive to this syndrome. This paper discusses the usefulness of a novel method to measure ionophore concentration in serum samples.

Monensin concentration in serum was evaluated in five- to six-week-old broad-breasted white turkeys kept in commercial facilities. Additionally, the liver vitamin E concentrations of these birds were evaluated. Vitamin E has been described to influence the signs of knockdown syndrome (5). The birds were allotted into three groups: 1) negative controls (healthy turkeys, fed a diet free of monensin), 2) positive controls (healthy turkeys, fed a diet that contained monensin), and 3) knockdown birds (turkeys diagnosed with knockdown syndrome, fed a diet that contained monensin). Monensin and vitamin E concentrations were determined in the feed samples from each ranch and group.

In turkeys from groups 1 and 2, histology of skeletal and heart muscles was normal, monensin was not detectable and vitamin E concentrations were within the normal expected concentration (3-15 ppm). Turkeys from group 3 had skeletal myopathy, but heart muscle was histologically normal. The serum monensin

concentrations in turkeys from this group varied from 0.05 to 11.5 ppb. Vitamin E concentration varied from low-normal to below normal. Monensin was not detected in feed from group 1, and it was below or within the approved use level (20-54 mg/kg) for turkeys in group 2. Monensin was below or within the approved use level in four cases (1.6-56 mg/kg), but above the approved concentration (145 and 171mg/kg) in two cases. Vitamin E was within the recommended range for turkeys this age in all feed samples.

These results suggest a correlation between the detection of monensin in serum, low concentration of vitamin E in the liver, and the presence of skeletal myopathy.

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THE EFFECTS OF VIRGINIAMYCIN IN IMPROVING RESPONSES OF LOW DOSAGE IONOPHORE FINISHER FEEDS

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SUMMARY

Finisher feeding programs involving low dosages of ionophores have become common in many production environments. A floor pen experiment was conducted to evaluate the efficacy of low dosage ionophore finisher programs against a late coccidiosis challenge (Day 29) and to determine whether virginiamycin (11 ppm) could improve performance under these conditions. Narasin (60 ppm) and salinomycin (44 ppm) produced significant reductions in coccidial lesion scores, although coccidiosis control was less than ideal. Similarly, final bird performance was improved by low level usage of salinomycin or narasin. When virginiamycin was added to feeds containing salinomycin, performance at 35 Days and final body weights (45 Days) were significantly improved compared to either ionophore fed alone.

INTRODUCTION

Producers often choose a low dose ionophore anticoccidial for the finisher feed to improve bird performance. The performance gain is generally attributed to the activity of the ionophore against coccidia which are present during an extended withdrawal period. Because the lowest approved dosages of these products are frequently employed, they may only provide control of minimal field exposures of coccidia. In situations where coccidial challenges increase, measurable effects of the infection become evident. Since one of the most common sequelae of coccidial infection is an elevated bacterial challenge, such exposures during the finisher phase of growth frequently affect final performance results.

The ability of virginiamycin to improve growth and nutrient utilization by poultry is well documented (1-9). Studies have demonstrated that in addition to its activity against *Clostridium perfringens*, virginiamycin improves performance results because it protects dietary energy and amino acids from microbial degradation, thereby allowing better use of the nutrients that are consumed (1, 3, 7-9). As a result of these properties, use of virginiamycin may be a valuable addition to feeds when broilers face a

bacterial challenge brought about by coccidial infection. Presented herein are the results of a recent study conducted to access the effects of virginiamycin in broiler chickens subjected to coccidiosis challenge and fed low levels of ionophores in the finisher feed.

MATERIALS AND METHODS

A 45-day floor pen study was conducted at Southern Poultry Research, Athens, GA to evaluate whether the inclusion of virginiamycin in finisher diets containing salinomycin would improve performance compared with narasin or salinomycin when fed alone. The experiment consisted of 36 pens, each starting with 60 male broiler chicks of the Cobb X Cobb strain. All starter and grower feeds were identical and contained salinomycin at 66 ppm and bacitracin methylene disalicylate (BMD) at 55 ppm. As a result, performance comparisons in this test began with the administration of finisher feed on Day 29, where four treatments were replicated in nine blocks of four pens each. The treatments were non-medicated control, narasin 60 ppm, salinomycin 44 ppm and salinomycin 44 ppm with virginiamycin 11 ppm. Thus, the low-level ionophores were included in the withdrawal feed (with and without virginiamycin) from day 29 through 45. On Day 29, all birds were challenged with a mixture of current isolates of *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* via the feed. The challenge level was titrated to allow a moderate to heavy coccidial infection to develop. On Day 35, five birds from each pen were selected, sacrificed, group weighed and examined for the presence of coccidial lesions. The upper, middle and cecal regions of the digestive tracts were lesion scored using the system of Johnson and Reid (10). Bird weights were recorded by pen at study initiation and at Day 28, 35 and termination (Day 45). Feed was removed and weighed at Day 28, 35, and at study end (Day 45). Upon assay, all feeds were found to contain the appropriate drug concentrations. All data were analyzed by ANOVA procedures contained in the Statistix® programs and significant mean separation was established with the LSD procedure at $P<0.05$.

RESULTS

During the first 28 days of the study, no statistical differences in bird performance were observed among treatments. In fact, growth and efficiency responses within and among treatments during this interval were surprisingly similar (Table 1). As a result of coccidiosis challenge at Day 29, all medicated treatments demonstrated improved performance compared to the non-medicated control by Day 35. While no statistical differences in performance were noted for the ionophores fed alone (salinomycin vs. narasin), the addition of virginiamycin (11 ppm) to finisher feeds containing salinomycin (44 ppm) provided performance results that were statistically better than salinomycin or narasin fed individually.

Table 1 also illustrates that by Day 45 the effects of coccidiosis challenge were still evident because all medicated treatments had significantly improved performance compared to the non-medicated control. As before, no differences between the ionophores (alone) were observed in final performance results. In addition, inclusion of virginiamycin to feeds containing salinomycin significantly improved final weights compared to all other treatments. Similarly, salinomycin/virginiamycin significantly improved feed conversion compared to narasin (alone) and the non-medicated control, but was statistically equivalent to salinomycin alone.

Coccidial lesion scores of all medicated treatments were significantly improved compared to the non-medicated treatment, although coccidiosis control was less than ideal for these treatments. Average reduction of lesion scores by these three treatments was only 29%. Salinomycin, with or without virginiamycin, significantly improved *E. maxima* lesion scores compared to narasin.

DISCUSSION

A strong coccidiosis challenge was induced at Day 29 in this study. Both narasin and salinomycin significantly reduced lesion scores, although coccidial infection was evident in all treatments. Under these conditions, performance was improved by low level usage of salinomycin or narasin at Days 35 and 45 of the test. However, finisher feeds containing salinomycin and virginiamycin had significantly improved body weights and feed conversion compared to all other treatments at Day 35, and provided significant improvements in final body weight when compared to narasin or the non-medicated control.

Because it is well recognized that coccidial infection is commonly followed by bacterial complications, the addition of virginiamycin to low level ionophore feeds may provide the bacterial control that produced significant improvements in bird performance.

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Table 1. Performance results of birds fed narasin, salinomycin or salinomycin + virginiamycin at Days 28, 35 and 45 (trial termination) in response to coccidiosis challenge at Day 29.

Treatment (ppm)	Day 28		Day 35		Day 45	
	F/G	Weight (kg)	F/G	Weight (kg)	F/G	Weight (kg)
Non-medicated	1.521 a	1.366 a	1.943 a	1.699 c	2.107 a	2.348 c
Narasin 60	1.520 a	1.366 a	1.863 b	1.759 b	1.985 b	2.451 b
Salinomycin 44	1.519 a	1.364 a	1.840 b	1.756 b	1.944 bc	2.441 b
Salinomycin 44 + Virginiamycin 11	1.521 a	1.369 a	1.810 c	1.783 a	1.911 c	2.521 a

Statistical differences for all data were established at $P<0.05$.
All treatments contained salinomycin 66 ppm + BMD 55 ppm from placement through Day 28.

THE EFFECT OF NATUSTAT® ON HISTOMONIASIS IN TURKEYS

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INTRODUCTION

Histomoniasis is caused by the protozoan parasite *Histomonas meleagridis*. While many gallinaceous birds can be infected, turkeys are especially susceptible. Morbidity and mortality in turkey flocks can reach 90% (5).

For a long time histomoniasis was no important problem thanks to separate rearing of turkeys and chickens, indoor rearing, and a variety of therapeutic and prophylactic drugs. However, in the last years several outbreaks of histomoniasis caused considerable economic losses, as outdoor rearing of poultry has been on the rise again and all therapeutic and prophylactic drugs were banned in the European Union (3). In the US only one feed additive for prevention of histomoniasis in turkeys is registered.

Currently the search for new antihistomonadal compounds is focused on "alternative" herbal products, which are used as feed aroma substances, and which are expected to meet good acceptance by producers and consumers.

The aim of this study was to investigate the effect of Natustat® after experimental infection of turkey poults with *H. meleagridis*. Natustat is a natural, plant-derived proprietary product for use in poultry feeds, which has been shown to posses some potential against infections with *Eimeria* and histomonads in broilers (1, 2).

MATERIALS AND METHODS

In this study turkey poults were treated with Natustat in the feed at levels of 250g (group 1), 500g (group 2), and 1000g (group 3) per ton feed, respectively. Treatment started on day 10 and lasted until the end of the study. Three of eight poults in each cage were infected intracloacally with cultured histomonads on day 14. The floor was covered with heavy paper for one week and the infection was allowed to spread to the other five birds (6).

The turkeys were weighed on days 10 and 14 and at the time of their death. Lesions in ceca and livers were scored on a scale between 0 (no lesions) and 4 (most severe lesions) (4). The amount of feed consumed was recorded.

Two weeks post inoculation the surviving animals were euthanized. Mortality, weight gain, feed conversion, and lesion scores in ceca and liver of the infected and treated groups were compared with a not infected, not treated group (group 4); a group that was infected and treated with nitarsone (group 5); and an infected, not treated group (group 6).

RESULTS

Mortality. In group 1 five birds died (three directly infected, two indirectly infected), in group 2 seven birds (four directly infected, three indirectly infected), and in group 3 seven birds (all directly infected). Eight directly infected birds of group 4 and two directly infected birds of group 5 died.

Weight gain. The mean weight gain per directly infected bird in the infected control (group 4) was 80g; in groups 1 to 3, 121g, 133g, and 213g respectively. The directly infected, nitarsone treated birds (group 5) gained 565g in average. The difference between group 5 and groups 1, 2, and 4 were significant.

The mean weight gain per not directly infected bird in the infected control (group 4) was 690g, in groups 1 to 3 550g, 621g, and 750g respectively. The directly infected, nitarsone treated birds (group 5) gained 885g in average and the birds not exposed to infection (group 6) 839g. The differences between the groups were not significant.

Feed conversion. The feed conversion rates were 3.15 in group 1, 2.92 in group 2, 2.37 in group 3, 2.43 in group 4, 2.28 in group 5, and 1.54 in group 6. The differences were not significant.

Lesion scores in the ceca. In the directly infected birds of groups 1, 2, and 4 the mean lesion score in the ceca was 4.0; in group 3, 3.89; and in group 5, 2.0. The mean lesion scores of indirectly infected birds in the ceca were 2.27 in group 1, 1.67 in group 2, 1.0 in group 3, 1.33 in group 4, and 0.07 in group 5. The differences between groups 1 to 4 were not significant.

Lesion scores in the liver. The mean liver lesion scores of directly the infected birds in groups 1 to 4 ranged between 3.44 and 3.67. The differences were not significant. The lesion score in the liver of directly infected, nitarsone treated birds was 0.67. In the indirectly infected birds the mean liver lesion scores in groups 1 to 4 ranged between 0.6 and 1.53. The differences however were not significant. The indirectly infected birds of group 5 showed no liver lesions.

DISCUSSION

Overall, Natustat had little effect against the severe lesions caused by direct inoculations of poults. However, liver and cecal lesions of the indirectly exposed birds were reduced and weight gain was increased after treatment with the higher doses of Natustat in comparison to the 250g/ton treatment. Comparison of treated groups with the untreated control group did not yield significant results. A major factor in this observation was the failure of histomonads to spread properly in two of three cages of this group. The disease infection model, which previously had shown good reliability for indirect exposure, is being reconsidered as a result of this data.

These results suggest that Natustat could be of value in reducing the spread of blackhead within a flock during an outbreak; however, more studies are needed to validate this.

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ROLE OF *DERMANYSSUS GALLINAE* IN THE TRANSMISSION OF *SALMONELLA GALLINARUM* IN CAGED LAYERS

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INTRODUCTION

The role of *Dermanyssus gallinae* (*D.g.*) as vector of various agents of infectious diseases has been emphasized by some authors, particularly in the past decades (1, 3, 4, 5, 6). The aim of this paper is just to give a contribution to the study on the role of such parasite in the transfer and maintenance of *Salmonella gallinarum* (*S.g.*) in a large caged layer farm.

MATERIALS AND METHODS

The farm under control consisted of about one million red-hybrid layers distributed in six houses. The mites were collected from four houses of the farm, where a variable infestation of *D.g* and a high mortality due to *S.g.* infection occurred, in spite of two or three vaccinations with the non-pathogenic *SG9R* (Intervet). A portion of parasites was suspended in saline, another washed two times with saline, then suspended in 4% formaldehyde solution for seven minutes (7). After centrifugation and removal of disinfectant and two further washings with saline, the mites were ground in a sterile mortar and resuspended in saline. The washing fluids, after treatment and suspension of mite tissue homogenates, were seeded in enrichment medium (selenite broth), then or directly in solid medium (Gassner). The identification of isolated microorganisms was done by biochemical and antigenic testing. The isolation of *S.g.* was performed weekly from the spleen of birds of six different houses with, or apparently without, mites.

The percentage of specific mortality due to *S.g*. from 18 to 60 weeks of age was calculated in the different houses on the ground of lesions weekly found at necropsy, often confirmed by bacteriological controls.

RESULTS AND DISCUSSION

The examined mites, obtained from four mites infested houses, resulted positive for *S.g*. The vaccination (also three times) demonstrated to be not sufficient to control the disease and prevent the transmission of *S.g*. in presence of mites in the houses. The contamination of outer and inner organs of the mites was demonstrated, so suggesting the localization of *S.g*. also inside the mite body. After treatment with formaldehyde the wash water resulted negative for *S.g*. The results of isolation of *S.g*. from parasites are reported in Table 1.

The transmission of *Salmonella* by arthropods, the mites mainly by bite, is a quite known phenomenon (2). It has been demonstrated that *S.g.* can survive in mites, even for 4 or more months after contact of them with the host (2). The spontaneous infection of mites population with *S.g*. shows the importance of a correct and continuous disinfection and disinfestation of poultry houses and farms for the successful control of *Salmonella* infection, also in case of specific vaccination of the birds. In fact, the vaccination proved to be rather effective, greatly reducing the incidence of

the disease, as observed in the houses without or with rare *D.g.* infestation.

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Table 1. Isolation of *Salmonella gallinarum* from *Dermanyssus gallinae* in different houses of the same farms from animals vaccinated three times with *SG9R* vaccine at 6, 17, and 27 weeks of age.

Age (wk)	House	Presence of mites	Mortality (%)		S.g. isolation	
			standard	due to S.g.	from mites (*)	from enlarged spleen
18 to 60	1	++	2.5 -4	2.2	+	+
	2	+++		28.0	++	+
	3	++++		30.0	++	+
	4	++++		36.5	++	+
	5	- or rare		0.7	-	+
	6	- or rare		1.2	-	+

(*) in inner organs of mites.

PRE-EPIDEMIC AND EPIZOOTIOLOGIC FEATURES OF AVIAN INFLUENZA IN NIGERIA

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SUMMARY

The pattern of the current H5N1 avian influenza (AI) epidemic and its management has revealed three major categories globally, namely: 1) countries with no outbreaks, 2) countries with continued outbreak management problems, and 3) countries with progressing outbreak management. One reference point in the third category is Nigeria where panic at the onset of the epidemic in 2006 gave way to more concerted responses and improved crisis management. This presentation describes the initial panic and the subsequent stable structure of Nigeria's epidemic preparedness and management. It also relates the reactions by stakeholders and provides an update on the epidemic profile and its management in Nigeria. Observations are highlighted on some roles of poultry and pigs peculiar in rural ecology with potential epizootiologic linkages to AI in birds and humans. Similarly, it touches on aspects of communal usage of village water ponds and poultry markets, which also pose grave possibilities in interspecies transmission of AI and public health.

INTRODUCTION

Following the maiden outbreak of the current H5N1 avian influenza (AI) epidemic from Southeast Asia in 2003, the epidemic has spread across many countries in continental Asia and Europe to Nigeria, Egypt, Niger, Cameroon, and Burkina Faso in Africa. The fact that the epidemic was reported in Nigeria ahead of some countries in Southeast Europe and North Africa was surprising and did not fit well into its traffic projections from Asia. Indeed, the multiplicity of the lineages of the H5N1 AI virus isolates from Nigeria (7) has stimulated a rethinking of the traditional roles of migrant birds and the introduction of AI into Nigeria. The option of eradication by slaughtering and the modalities for compensation have been applied with wide variances in affected countries. These and other unresolved issues are part of the complications which perhaps account for success or failure in the control and management of the epidemic. However, the fact that a re-emerging epidemic like AI is this problematic, perhaps implies that sufficient lessons have not been learned from previous epidemics, to guarantee an adoptable or at least adaptable framework for its efficient management in many affected countries. A more open-ended global information framework in the current episode is therefore a necessity for enriching the capacity for the management of the current H5N1 bird flu epidemic and preparedness for the future epidemics. This presentation from the experiences on developments in Nigeria – a leading poultry producing country in Africa – therefore represents a noteworthy component of such enrichment.

PRE-EPIDEMIC BACKGROUND IN NIGERIA

Avian influenza is a reportable list-A disease but it remained only a poorly recognized entity in the country until recently. However, there have been at least some subtle warning signals of its activity in the country including the serologic evidence of H1NI and H5N1 before the current epidemic. (4, 10). These pre-epidemic findings were not accorded conventional publicity in Nigeria, mainly because AI unlike Newcastle disease, bursal disease, and Rinderpest, was never a frontline problem in Nigeria's poultry. This scenario represented the general level of awareness until about mid-2004 when our study team at University of Ibadan, was lead by a concern for the poultry industry and a focus on the global trend of the H5NI epidemic, to draw the attention of the government to the need for vigilance and national preparedness.

This initiative therefore preceded a number of statutory and related activities by stakeholders in the country. Examples of such activities are as follows:

By FGN: a) Inter-Ministerial Committee on Flu based in the Ministry of health in the third quarter of 2005; b) The Technical Advisory Committee on AI in the Ministry of Health, December 2005; c) Technical Committee of Experts, in Fed Livestock Dept., December 2005.

These two technical committees which were primarily concerned with the health and agricultural

sectors respectively, promptly submitted separate preparedness documents to FGN in December, 2005.

ABU/FVM: A Colloquium on Bird Flu in Ahmadu Bello University, Zaria, invited a multi-disciplinary team of experts to make contributions which were published with a copy sent to the Hon Minister of Agriculture in December, (3).

These developments and the increased publicity very rapidly lead Nigeria from a state of relative un-awareness to a series of activities in preparedness, planning and mobilization through public and private media.

PREPAREDNESS PLANS IN NIGERIA

Although the two earlier mentioned technical committees submitted what could be regarded as good preparedness plan dossiers, which included operational logistics and material requirements to FGN promptly in Dec 2005, the arrival of the epidemic so soon after in January 2006, appeared to have caught the country by surprise. There was hardly enough time to study the preparedness documents or adopt and procure logistic requirements. The requisite training of field staff and other stakeholders could hardly be done and so, panic was therefore inevitable in the circumstances. However, with the rather emphatic policy stance of FGN on the emergency, national and international efforts were speedily mobilized to put in place a concerted response program to replace the initial panic. The proficiency of the poultry veterinarians in the early and correct field diagnosis of the disease in January 2006 before the confirmation from Italy three weeks later, made the difference. The salient lesson for other countries is that the absence of the field diagnosis and the follow-ups in January could have obscured, or at least delayed, the recognition and confirmation of the epidemic in Nigeria.

REACTIONS AND RESPONSES TO THE EPIDEMIC

Soon after the report of the outbreak in Nigeria, there was widespread reaction and panic which seemed to drown the incipient preparedness efforts. The basis of the panic varied and included the following examples:

- There was palpable fright and frustration among investors in view of economic implications on poultry production.
- Small scale (rural poultry) operators were scared by the danger of losing source of family livelihood with attendant aggravation of poverty.
- Large commercial operators bemoaned the looming loss of their investment capitals.
- Employees were frightened by their helplessness in the face of impending lay-offs.
- Consumers progressively abandoned patronage of poultry meat and eggs and opted for fish, beef and other substitutes, with cost implications, as the demand pressure forced prices of beef and fish upwards.
- Producers and marketers of poultry products lost a good chunk of sales and incomes.

The precise and quantitative details of these problems are still to be fully documented or published.

There were also some less logical problems, including the covert disposal of chickens from flu-affected sites by quite a few economically deprived people. In all these, there was a good level of enlightenment and publicity campaign by FGN to restore order and reduce panic. It was therefore obvious that the Nigerian public, including the rural dwellers, was progressively sensitized on the challenges and preventive cautions involved.

The responses consisted not only of the general stereotypes but also customized components to suit the local demands. FGN proclaimed a slaughter and eradication policy for the stamping out of the flu epidemic. Vaccination was therefore not officially adopted but some individual attempts to vaccinate poultry flocks were alleged. Public health measures for poultry workers and veterinarians, family and children, hospital workers, and poultry product consumers were canvassed.

The epidemic which had spread rapidly in the first quarter of the year from only two states (Kaduna & Kano) in February to nine states in the second quarter posed grave challenges and fear to the public and government. As at October 2006, about 16 states touching all geopolitical zones of Nigeria had reported the epidemic in commercial and/or rural poultry. The hub of commercial poultry industry, in south-west Nigeria, reported outbreaks in some medium to large stock holdings (of over 200,000 birds in some cases) in the second quarter. This rapid spread persisted despite the slaughter and quarantine measures for its eradication. It then slowed down conspicuously later in the second quarter which coincided with the ingression of the hot season. An active disease surveillance plan has now been evolved with international support, to more closely and comprehensively monitor the status and movement if any, of the epidemic in the country. Sporadic outbreaks are still being reported from a few locations in the southeast and northeastern axis of the country.

It is noteworthy that outbreaks of H5N1 AI were reported from neighboring Niger, Burkina Faso, and

Cameroon, a few weeks after its first appearance in Nigeria. While it is now obvious that Nigeria represents an example of some measure in transparency in the reporting and management of the epidemic, there is justifiable cause for concern in some other countries with doubtful sense of transparency on the epidemic despite its tremendous global stakes.

COMPENSATION PROGRAM

The slaughter policy adopted for the eradication of the disease in Nigeria necessitated a contingent compensation program. The objective was to help ameliorate the losses without pretext to full scale pay-back of costs of the slaughtered birds to their owners. A compensation at the rate of N250 (about 2USD) per chicken, the equivalence of about 40% market value, was granted by government; with the promise of assistance in re-stocking. This policy generated mixed reactions amongst stakeholders and perhaps precipitated incomplete compliance by some of them. Table 1 presents the summary of the pay-outs as mid-2006, in this program. A similar program in Niger Republic, to the north of Nigeria, offered the equivalence of over 100% market value of each slaughtered chicken to farmer! This is a noteworthy contrast from two neighboring countries but the anticipated volume of compensation may have played a decisive role in the shape of the policy. The size of the total poultry population in Niger is about 15 million or less that 10% of Nigeria's total stock. All said, these situations can not but impact significantly on the compliance by stock owners, with slaughter and eradication program. It is therefore a matter for more global attention, especially as the epidemic itself is primarily a global problem which should merit not only international collaboration but also funding. The future should also demand some more internally driven efforts by individual nations to promote a more virile poultry and livestock insurance policy which can come readily handy in the face of massive crisis like the one posed by the current AI epidemic.

PUBLIC HEALTH CONNECTIONS IN RURAL LIVESTOCK HUSBANDRY

Although the huge losses in poultry stocks make the current epidemic, one of the most serious agricultural problems worldwide, it is also an important zoonosis (*zooanthroponosis* or *ornithoanthroponosis*). One main cause for concern about the H5N1 avian flu is in the evidence that it has crossed the species barrier. It is known that pigs occupy a strategic platform for mutation of AI virus. It has been explained (11) that the presence of both the α2,3 and α2,6 sialic acid receptors in pigs epithelia, makes it an auspicious receptacle for mixed infection by avian and human flu viruses. In the typical rural and peri-urban settings in Asia and Africa, it is known that humans live in close proximity with their poultry, while poultry and pigs both share the same or close husbandry ecology. It is not unusual in the deep rural settings for poultry to enter the abodes of their keepers for food crumbs in the day time or for rest at night time.

Children are often fond of pet-plays with family chickens while marketers do not only handle live chickens closely and regularly but are indeed known to lay their tired heads on the chicken baskets for brief naps during day-long market periods. These are excellent scenarios for the transmission of LPAI or even HPAI zoonosis. All that is needed for the completion of the scary bird/human/pig epizootic triangle is the inevitable day time ecologic interaction between chickens and pigs on the communal refuse dumps during foraging and scavenging, which are the daily past times of these two common rural and family livestock.

These observations are indeed ominous signals of what could happen along the bird flu and human pandemic pathway. This perhaps offers part explanation to the pattern of spread of the current bird flu epidemic to humans in Asia through the roles of backyard livestock husbandry. There is no full analogue to this in the industrial poultry husbandry where the farm operators, especially the poultry stock and meat handlers, may be exposed to AI infection.

The major difference is that this is limited to a two-way interaction with little or no direct porcine (third party) complement of the epizootic triangle for the possible generation of mutant flu viruses. Aspects of such occupational considerations have been recently alluded to by Hayden & Croisier (9).

There is yet another noteworthy epizootiologic connection in the use of open rural (untreated) water ponds for swimming, by rural dwellers. It is common knowledge that many of such pools are frequented by water- or wild birds and rural poultry which contaminate the water with their fecal droppings and oral discharges. Such contaminated water ponds can serve as potential source of bird flu infection through contact with human eyes and conjunctiva during swimming. Although they have so far not been clinically associated with the sources and spread of avian flu in Africa, the technical basis mediated by the mucosal α2,3 sialic acid receptors in human eyelids have previously been established (6, 9).

The variety of epizootiologic situations so far illustrated here, are obviously more related to Africa and Asia than to the developed countries of Europe and America. The puzzle however is, with the exception of

the isolated cases in Egypt, there has been no case of such human infection reported in Nigeria and most African countries where bird flu has been reported in poultry. These and other similar puzzles in the bird flu situation in Africa raise questions which call for the application of empirical research as means to the understanding of the ramifications of the epizootiology of AI in Africa. Some crucial hints along this line comes from the restricted host and virus-subtype interactions for example, although pigs are experimentally susceptible to infection with virtually all subtypes, only two HA and NA subtypes (H1, H3, N1, & N2) have previously been recorded in natural infection of pigs. Such restrictions as well as husbandry and environmental factors, especially temperature, may be involved in Africa's avian flu epizootiology and perhaps help to explain some of the peculiarities highlighted in this article.

SOURCE OF AI IN NIGERIA AND MANGEMENT OPTIONS

As of January, 2006 when AI was diagnosed in Nigeria, there was doubtful, if any, evidence of the arrival of migrant birds in the country. A report from the Bird flu Colloquium organized in the Faculty of Veterinary Medicine at Ahmadu Bello University (ABU), Nigeria in 2005, speculated on illegal importation of live poultry and products, as well as the activity of resident flu virus pathotypes as possible sources. Indeed, considering previous serologic findings of H1N1and H5N1 infections in Nigeria's poultry and pigs (4, 10), which serve to confirm older preliminary unpublished records on bird flu in 1984 and 1986, it seems impossible to rule out low grade flu virus activities in Nigeria's industrial and rural poultry, which predates the current H5N1 epidemic. These could have been an ingredient in the generation of AI virus activity over the years in Nigeria. The recent publication on three or more lineages of H5N1 virus with rather independent sources of entry in Nigeria (7) seemed to have confirmed not only the ABU report but also this hypothesis. In which case, any contribution from migrant birds could not have been immediate but would appear to have built up over a period before 2006.

With all these varied backgrounds to the profile of Africa's AI epidemic, including the complex mixture of industrial and rural poultry production systems, it is logical to also expect that the management and control of the epidemic in Africa will demand a complex or combined regional approach. A concerted stamping out policy has a good place in the control of an epidemic like AI but the local or regional peculiarities, such as ecology, geography, poultry husbandry, inherent technologic facilities and know-how, systems operative efficiency, etc., are the determinants of the relevant and achievable disease control program. Even in advanced technologies, we can see the example of differences in the customization of control policies; like in the partial or full adoption of poultry vaccination by France, Italy, Holland, and Hong Kong (5, 8; Peiris, 2006) versus the apparent exclusion of vaccination in some other European countries and USA. There are arguments for and against the adoption of vaccination for the control of bird flu in poultry. From a comprehensive point of view, vaccination of poultry is at the least one good way to minimize the spread of *the full blown* epidemic in a *nebulous husbandry* and ecology as in Asia and Africa, while also ensuring that the industry and the millions of problematic pockets of rural poultry survive. It is however crucial in the circumstances to carefully define the criteria and logistics for such selective program. In all these, the economic and epizootiologic importance of the typically small family/rural poultry flocks as explained by Adene (1) will demand some special considerations in terms of dose-package, shelf life or stability, route of application, and even the accessibility to such range-inclined and nebulous foci of mini-flocks.

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Table 1. FGN's allocation on avian influenza.

S/No.	States	State poultry population (2005)	No of farmers	No. of LGA affected	Type of birds	Bird population of the affected farms	No. dead	No. depopulated	Amount for compensation (₦)
1.	Anambra	82,876	1	1	L	500	500	0	
2.	Benue	1413402	2	1	L/Hy		NA	NA	
3	Bauchi		6	2	Layers	147,782	63,654	84,128	21,032,000.00
4.	Abuja, FCT	45,205	3	3					
5.	Kano	226,024	58	10	Mixed	---	77,465	143,375	35,638,250.00
6.	Kaduna	188,353	59	3	Mixed	113,798	76,149	37,303	13,481,500.00
7.	Katsina	180820	4	2	Layers	N/A	N/A	4,071	1,017,750.00
8.	Lagos	663,100	1	1	Layers	18,050	14,361	3,689	942,250.00
9.	Nassarawa	650,948	517	1	L/Hy	9,817	1055	8306	3,141,000.00
10.	Ogun	403899	1	1	Layer	125,000	39,793	85,210	21,304,750.00
11.	Plateau	789,576	13	2	Layer	54,358	12,053	42,305	10,576,250.00
12.	Rivers	206,985	1	1	Layer	N/A	N/A	N/A	
13.	Yobe	182,957	2	2	Layer	N/A	N/A	N/A	
								331,865	

Source FLD Nigeria, 2006

EXPERIMENTAL INOCULATION OF PIGEONS *(COLUMBA LIVIA)* WITH TWO NEWCASTLE DISEASE VIRUS STRAINS ISOLATED FROM COMMERCIAL POULTRY IN MEXICO

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INTRODUCTION

The velogenic form of Newcastle disease (vND) is one of the most important viral diseases of birds worldwide and produces a vast global economic impact (12, 17, 18, 25). Outbreaks of vND are obligatorily reported to the World Animal Health Organization (OIE) (2, 4, 6, 12, 18, 26). In Mexico, there is government surveillance for vND and official procedures have been established for the control and eradication of this disease NOM-013-ZOO-1994 (17).

Newcastle disease is caused by a Paramyxovirus 1, which characteristically consists of a single molecule of single-stranded RNAs. Velogenic strains include basic amino acids in the F site cleavage (1, 4, 14). All velogenic and mesogenic strains have phenylalanine at the residue 117 (4, 18, 26). The virulence of the different Newcastle disease virus (NDV) strains is highly variable depending of the host. Chickens are highly susceptible, but pigeons (*Columba livia*) may be infected and show few or no clinical signs, even with lethal strains for chickens. The role of pigeons and other birds in vNC's spreading has been discussed (1, 2, 3, 4, 5, 6, 7, 8). There are not previous studies concerning the effects of experimentally infected pigeons with vND strains (velogenic and mesogenic) isolated from Mexican commercial poultry. Direct or indirect contact between pigeons and poultry occurs frequently. The objective of this work was to describe the clinical, pathological, and immunological effects of pigeons experimentally infected with two different NDV strains (a velogenic and a mesogenic strain) at different days post infection.

MATERIALS AND METHODS

Two pathogenically different NDV strains isolated from Mexican poultry were used that were previously characterized as velogenic and mesogenic (Ortiz, 2003). These viruses have titers of $EID_{50\%}10^{6.5}$/mL in SPF chicken embryos. Sixty racing pigeons younger than three years old and serologically negative to NDV by hemagglutination inhibition test (HI) were used. The pigeons were distributed in three groups and were maintained with water and food *ad libitum* in a high security facility of the Avian Medicine Department (College of Veterinary Medicine, National University of Mexico).

Experimental infection. The first group of pigeons was inoculated with 0.1 mL of a NDV velogenic strain. The second group was inoculated with 0.1 mL of a NDV mesogenic strain. Intraocular route of inoculation was used in both cases. The third group was a non-inoculated negative control (7, 17, 24).

Tracheal and cloacal swabs were collected for virus isolation at 24 and 48 hours after inoculation (17).

Pigeons were observed every eight hours post infection for the detection of clinical signs or mortality. In order to analyze the antibody response, serum samples were taken at 7, 14, 21, and 28 days after inoculation and measure by HI test.

Five pigeons from each group were euthanized at 7, 14, 21, and 28 days post inoculation. Complete necropsies were performed and samples from lungs, trachea, spleen, cecal tonsils, and brain were taken for virus isolation in SPF chicken embryos and histopathology.

Gross and microscopic lesions were described.

RESULTS

1. Clinical signs characterized by respiratory distress, diarrhea, anorexia, and bristly feathers were observed in infected pigeons with both viral strains.
2. Mortality was not observed.
3. Both, velogenic and mesogenic strains were isolated from several organs at 7, 14 and 21 days post inoculation.
4. A measurable antibody response was found in pigeons inoculated with both strains at 7, 14, 21 and 28 days post inoculation (17, 22).

CONCLUSIONS

1. Pigeons were infected with velogenic and mesogenic virus strains isolated from commercial Mexican poultry.
2. The infected pigeons exhibited clinical disease but not mortality, and were able to recover from the infection.
3. Virus isolation from different organs was possible at 7, 14 and 21 days post infection.
4. Pigeons are potential carriers of Newcastle disease virus strains and could spread the disease in commercial poultry and in backyard poultry.

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PERFORMANCE COMPARISON BETWEEN THE USE AND NON-USE OF AN ENTERIC HEALTH ANTIBIOTIC PROGRAM IN COMMERCIAL BROILER FLOCKS

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SUMMARY

Over the past decade, the methods for using antibiotics in poultry production have changed (3). These changes are due to the increasing microbial resistance to the antibiotics used in poultry production and their potential for leading to human health problems. The elimination of using antibiotics for prophylaxis began in 1986 in Sweden (1) and quickly spread across the European Union (2). In the US, there has been little regulatory activity regarding the use of antibiotics. However, current trends show that enteric health antibiotics are being removed from broiler diets across the country. This is largely due to consumer pressure on the poultry industry to remove antibiotic growth promoters form animal feeds. This pressure is affecting commerce so that major retailers are not accepting poultry that have been feed antibiotics in the feed (2).

An experiment was conducted to compare the differences in performance parameters between broilers that were fed enteric health antibiotics in the diets and broilers that were not fed enteric health antibiotics in the diets. This is the preliminary report from four flocks of a five flock trial. Broilers were reared under commercial settings in solid-side wall, tunnel ventilated broilers houses. Initially, 27,600 broilers were placed per house and reared for 49 days. The four-house farm was divided into two separate farms with two houses being fed antibiotic growth promoters (Trt 1) and the other two being fed a naïve feed (Trt 2). All birds were fed commercially produced starter, grower, and withdrawal rations. All starter and grower diets fed to both treatments contained the current coccidiostat being used by the respective commercial poultry integrator. Individual body weights of 100 randomly selected birds per house were collected at 18, 35, and 49 days of age and average body weight was calculated. Average body weight, feed conversion, and adjusted feed conversion were calculated and examined for each farm at 49 days of age. Coccidiosis lesion scores using the Johnson and Reid Method were collected at 14, 21, 28, 35, and 42 days of age. At the conclusion of each flock 140 birds from both treatments were selected and processed in a yield study. All statistical differences were considered significant at $P<0.05$ using SAS statistical software (SAS Institute Inc., Cary, NC).

During the first two flocks, Trt 2 had a significantly higher average body weight at 18 days of age as compared to Trt 1. By the third flock, no significant difference was detected between Trt 1 and Trt 2 at 18 days of age. At 35 and 49 days of age, there were no significant differences between the two treatments during the first three flocks, respectively. Trt 2 had a higher average body weight and an equal or lower feed conversion than Trt 1 at the end of flocks 1 and 2. At the conclusion of flock 3, Trt 1 had a higher average body weight and a lower feed conversion as compared to Trt 2. Average body weight, feed conversion, and adjusted feed conversion can be seen in Table 1. Coccidiosis lesion scores increased significantly from flock 1 to flock 3, respectively. *Eimeria acervulina* and *E. maxima* lesions were detected in both treatments at 14 and 42 days of age during the flock 1. During flock 2, *E. acervulina* and *E. maxima* lesions grew progressively worse for both treatments from 28, 35 and 42 days of age, respectively. Trt 2 exhibited more total lesions than Trt 1 at 28 and 35 days, during flock 2. At day 42, Trt 1 had more total lesions than Trt 2 for *E. acervulina* and *E. maxima*. Some slight enteritis was detected and a majority of the birds suffered from femoral head necrosis for Trt 2 from flock 2. Lesions were detected in both treatments at 21, 28, 35, and 42 days of age for flock 3. *E. acervulina* and *E. maxima* lesions progressed in severity from week to week. More *E. acervulina* and *E. maxima* lesions were detected in Trt 1 than in Trt 2 throughout flock 3.

The results of the yield study for each flock are as follows. Trt 2 had a significantly greater breast meat yield than Trt 1 for flocks 1 and 2. Breast meat yield was not significantly different between the treatments for flock 3. Tenderloin yield was not significantly different between the treatments for flock 1 and 2, but Trt 2 had a significantly greater tenderloin yield than

Trt 1 for flock 3. Total white meat yield was significantly greater for Trt 2 during flocks 1 and 2. For flock 3 there was not significant difference seen for total white meat yield between the treatments.

Flock 4 is currently being reared and all pertaining data will be presented at the conference. The last flock of this study will conclude in May 2007.

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Table 1. Average body weight, feed conversion, and adjusted feed conversion for Trt 1 and 2 at 49 days of age.

	Flock # 1		Flock # 2		Flock # 3	
	Trt 1	Trt 2	Trt 1	Trt 2	Trt 1	Trt 2
Average body weight[A]	2.62	2.66	2.60	2.65	2.62	2.59
Feed conversion	1.83	1.80	1.81	1.82	1.90	1.94
Adjusted feed conversion	1.62	1.59	1.61	1.61	1.69	1.74

[A] Average body weight is in kilograms.

BLINDNESS DUE TO RETINOPATHY IN GUINEA FOWL

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ABSTRACT

Blindness was observed in 10 day-old Guinea fowl chicks. The incidence during the first week after its appearance ranged from 25 to 40% in a population of 100,000 Guinea fowl raised on different farms.

At the beginning of the syndrome the clinical signs of blindness in birds included aimless wandering and increased mortality rate due to starvation (2-3% over the average). The birds lacked pupillary reflexes to light and there were no gross lesions in the eyes. Histologically there was mild to moderate disorganization and disruption of the retinal layer, mild to moderate vacuolation and degeneration of photoreceptors, moderate multifocal retinal detachment with eosinophilic proteinaceous material, occasional rosette formations. The Guinea fowl chicks came from three different breeder sources but all the birds were given the same feed. The condition was not observed in the subsequent flocks that came from the same breeder sources but were given different feed. Based on these observations toxicity of feed is suspected as the cause of blindness in the Guinea fowl.

(A full length article is in preparation for publication.)

PHYLOGENETIC ANALYSIS OF VERY VIRULENT, CLASSIC AND VARIANT INFECTIOUS BURSAL DISEASE VIRUSES FROM CENTRAL AND SOUTH AMERICA

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INTRODUCTION

Infectious bursal disease viruses (IBDV) are present wherever chickens are being commercially raised. Classic virulent (cv)IBDV can cause low levels of mortality (5-10%) from clinical infections, usually after three weeks of age. In the late 1980s very virulent (vv)IBDV was first seen in European flocks suffering much higher mortality (50-60%) from acute clinical disease. At about this same time, antigenic variant (v)IBDV was being isolated from U.S. broiler flocks that were being infected by seven to ten days of age despite having high (classic) maternal antibodies. These flocks showed no outward appearance of being infected, like one sees with cvIBDV infections, but they commonly suffered from sequellae of immune suppression – high mortality from secondary *E. coli* infections, gangrenous dermatitis, generally poor performance, etc. This became termed as "subclinical" IBD.

In the past several years, PCR-RFLP analysis of the variable region of Viral Protein 2 (VP-2) has suggested the presence of variant IBDV as well as vvIBDV in various Latin American countries. We have conducted several field surveys, mostly from problem farms suffering from either clinical IBD mortality (as high as 60%) or chronically poor performance and secondary diseases. The IBDV samples recovered from these cases were submitted to Daral Jackwood for PCR-RFLP followed by amino acid sequencing analysis. The results of this survey are presented in the accompanying figure.

MATERIALS AND METHODS

Chickens from farms with a history of either high clinical mortality or poor performance and secondary disease problems were selected for bursal health surveys. Most samples were collected consecutively at 3, 4, and 5 weeks of age to find the correct window of IBDV infection. Some flocks were sampled as early as two weeks of age and some were sampled only once (breaks of clinical IBD). Bursas were split in half so that paired samples could be either formalin-fixed (for imaging analysis) or phenol-chloroform inactivated (for importation and PCR analysis). Over 50 field samples were positive for IBDV RNA that could be analyzed. A phylogenetic tree was constructed using software (UPGMA) that calculates the relative bootstrap values of all entries. For further clarification, IBDVs were also bracketed to the right and the relevant amino acids of each grouping or cluster are summarized.

RESULTS

Analysis of the field samples reveals six general clusters of IBDVs:

- Very virulent IBDV from Bolivia, Brazil, Colombia, El Salvador, and Venezuela
- Classic virulent IBDV from Argentina, Bolivia, and Mexico
- Classic virulent IBDV (S-222) from Argentina and Colombia
- Classic IBDV with minor variant traits from Venezuela and Columbia
- Variant IBDV from Bolivia, Colombia, Ecuador, and Mexico
- Variant IBDV with minor classic traits from Mexico

DISCUSSION

Most vvIBDVs have a characteristic VP-2 phenotype that distinguishes them from cvIBDV. These unique amino acid substitutions are underlined in the figure. It is not known for sure whether any of these substitutions are requirements for the expression of high clinical mortality – there are several exceptions, including three in this survey: two El Salvador IBDVs (lacking S299) and perhaps Bolivia isolate 05B62 (lacking I242, I256 and S299). Indeed, VP-1 most likely plays a critical role in hypervirulence as well. However, the highly conserved nature of the vvIBDVs makes sequencing analysis of VP-2 a valuable diagnostic and epidemiological tool.

The classic virulent IBDVs fell into three separate clusters (2, 4, and 5 in Figure 1). The classic antigenic type is largely determined by the immunodominant hydrophilic peak B, which houses G318. Cluster #2 contained classic viruses with the typical classic amino acid sequences. IBDVs in Cluster #4 are considered classic viruses, but with features of typical U.S. variants in minor hydrophilic peaks 1 and 2. This pattern is similar to the Lukert viruses (i.e., Bursine-2), which are also classic viruses with some features of variants in the minor peaks. Cluster #5 contained classic viruses with a substitution of serine at amino acid position 222. Some classic viruses from Europe as well as the Lukert viruses also have S222.

The variant IBDVs were seen in two different clusters. Cluster #3 contained variant IBDVs that shared the same key amino acid changes as the vast majority of U.S. variant field viruses (see Figure 1). In fact, two reference U.S. variants, Delaware E and Fort Dodge's Group-6 inactivated vaccine virus, grouped into this variant family cluster. Cluster #6 contained viruses with variant features in major peaks A and B (T222 and D318, respectively), but with classic "looks" in the two minor peaks. These variant viruses are interesting because many of the unique sequences we recently analyzed in Europe had these same characteristics.

To summarize, we sequenced IBDVs from a total of eight countries in Central and South America, including Argentina, Bolivia, Brazil, Colombia, Ecuador, El Salvador, Mexico, and Venezuela. We found sequencing patterns consistent with vvIBDV in Bolivia, Brazil, Colombia, El Salvador, and Venezuela and variant viruses in Bolivia, Colombia, Ecuador, and Mexico. Most of the classic/variant hybrids (cluster #4) were seen in Venezuela. Argentina samples only yielded classic sequencing patterns.

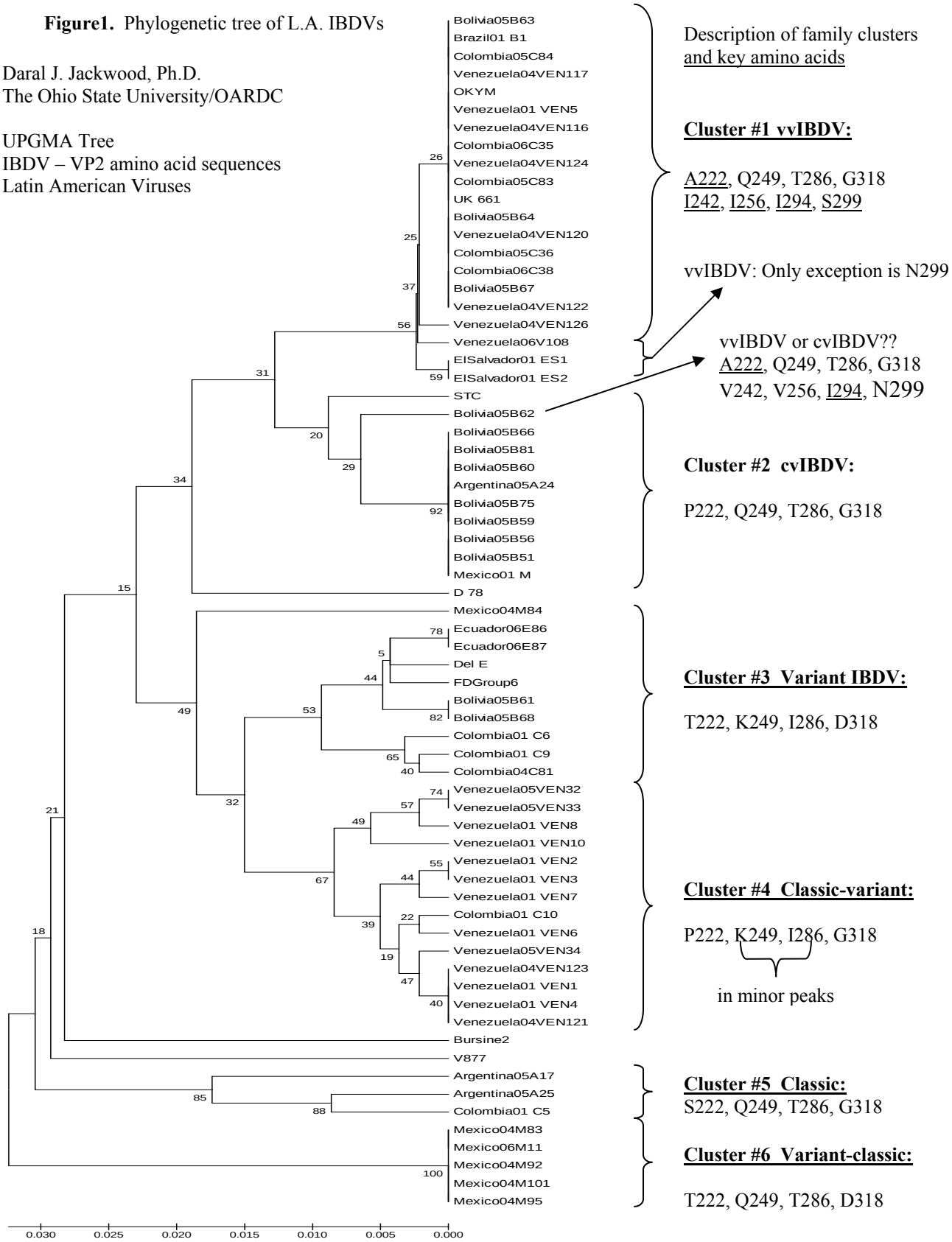
This survey thus demonstrates the presence of variant IBDV across a wide stretch of Latin America. This is perhaps not such a surprise, especially in areas that have seen the benefit of using bursal-derived killed vaccines, which offer better variant protection. Since the discovery of vvIBDV in Brazil at the turn of the century, it is apparent that this virus is spreading to various other Latin American countries.

Strategies to control vvIBDV are understandably much different than our current practices in the United States. The presence of vvIBDV as far north as El Salvador should be a wake-up call not only for Mexico but also for those of us north of the border.

Figure1. Phylogenetic tree of L.A. IBDVs

Daral J. Jackwood, Ph.D.
The Ohio State University/OARDC

UPGMA Tree
IBDV – VP2 amino acid sequences
Latin American Viruses



Description of family clusters
and key amino acids

Cluster #1 vvIBDV:

A222, Q249, T286, G318
I242, I256, I294, S299

vvIBDV: Only exception is N299

vvIBDV or cvIBDV??
A222, Q249, T286, G318
V242, V256, I294, N299

Cluster #2 cvIBDV:

P222, Q249, T286, G318

Cluster #3 Variant IBDV:

T222, K249, I286, D318

Cluster #4 Classic-variant:

P222, K249, I286, G318

in minor peaks

Cluster #5 Classic:
S222, Q249, T286, G318

Cluster #6 Variant-classic:

T222, Q249, T286, D318

THE INFLUENCE OF *E. COLI* INOCULUM TITER AND VIRALLY INDUCED IMMUNE SUPPRESSION ON THE INCIDENCE OF CELLULITIS IN A BROILER SKIN CHALLENGE MODEL

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INTRODUCTION

Escherichia coli induced cellulitis, also called infectious process (IP), is one of the leading causes of broiler condemnations at processing. The generally accepted mechanism for the development of cellulitis is via skin scratch inoculation. Thus, preventative measures focus on management techniques that diminish the incidence of scratches. Proper litter management can also influence the challenge load of resident bacteria. As with any infectious disease, the natural immune status of the bird can potentially play a role as well. Using a previously established skin challenge protocol, our goal was to see if the *E. coli* challenge load and/or the flock's immune status could affect either the incidence or severity of cellulitis lesions in a commercial broiler flock.

MATERIALS AND METHODS

A commercial broiler flock was housed in Horsfall isolator units (15 per) at Auburn University. Half of the birds received a combination of challenge viruses: reovirus 2408 strain intratracheally (IT) and CAV intramuscularly (IM) at day 3; and IBDV-Del-E and AL-2 (eye/nose drop) at 7 days of age. At 25 days of age the two treatments were further subdivided according to the following *E coli* challenge treatments, using isolate 29A: 1) no challenge, 2) subcutaneous (SQ) injection of 0.1 mL over the right breast muscle using a high dose (4.7×10^8 CFU) and 3) SQ-low dose (4.7×10^5 CFU). *E. coli* 29A is a serotype O78 that was isolated from an Arkansas broiler flock that suffered from high IP condemnations.

Ten birds were bled at hatch to determine their maternal immune status to reovirus, IBDV, and CAV using Idexx ELISA. Fifteen others per control group were weighed and posted at 18 and 25 days to measure the "take" of the viral agents given. At 32 days of age (7 days pch) all remaining birds were weighed and posted. Cellulitis lesions were scored using criteria previously established by Macklin and Norton: Grade 0 → no cellulitis lesion; Grade 1 → mild-focal (<2cm in diameter); Grade 2 → moderate-focal (cellulitis covers up to a quarter of the breast muscle); Grade 3 → moderate-diffuse (cellulitis covers half the breast muscle; there is severe muscle infiltration); Grade 4 → severe-diffuse (entire breast muscle is covered with cellulitis; severe muscle infiltration).

RESULTS

Viral Challenge

Reovirus. The flock's day of hatch geometric mean titer (GMT) to reovirus was very low (785) and a high percentage of birds were in 0-1 titer groups (60%). The 32 day mean body weights of all virally challenged flocks were very similar and were significantly lower ($p<0.05$) than their non-virally challenged cohorts. Body weight suppression of virally challenged groups ranged between 7.2% and 12.7%. While the Hi-dose *E. coli* group had the highest body weight suppression, this difference was not significant.

CAV. All chicks tested strongly positive for CAV antibodies (ELISA). The challenge controls showed no thymus depletion at 18 and 25 days of age and only modest depletion in some birds by 32 days (see Table 1). Pooled thymus samples were negative for CAV PCR at 18 days of age (data not shown). 25-day samples were not tested but a percentage of samples in each of the three virally challenged groups were PCR positive at 32 days of age.

IBDV. The ELISA GMT was 7,749 with a coefficient of variation of 28.2%. At 18 days of age 58% of the challenge controls were histologically intact based on bursal imaging, compared to 0% intact at 25 days and 10% at 32 days of age (see Table 1). The percentage of intact bursas in the virus + *E. coli* groups was 11% and 18% at 32 days of age in the SQ-Hi and SQ-Lo groups, respectively.

E. coli *Challenge*

Without the viral pre-challenge, only the SQ-Hi dose group contained birds with cellulitis (91%), as seen in the table. Comparing groups receiving the viral

pre-challenge to those that received only *E. coli*, there was no difference between SQ-Hi groups on lesion scores (both 2.2) or incidence (91% vs. 75%, respectively). There was a difference in the SQ-Lo groups, however, as only the group pre-challenged with the viruses had cellulitis lesions (36%). No internal *E. coli* lesions were observed, indicating that the disease was contained to under the skin.

DISCUSSION

Due to very low reovirus antibodies at hatch, this commercial broiler flock was susceptible to reovirus at the time of challenge (three days), which likely led to the significant body weight suppression (we reported similar findings in WPDC 2006). However this flock's maternal immunity to IBDV and CAV was high enough to delay the IBDV infection until about two weeks of age (based on bursal imaging—not shown) and the CAV infection until about four weeks of age. While early reovirus infections can cause varying degrees of immune suppression, it is quite possible that the two-week IBDV infection and perhaps even the late CAV challenge also contributed to the compromise in *E. coli* immunity seen in this study. Generally, IBDV infections before two weeks of age can cause profound, permanent immune suppression while infections after two weeks are less damaging and temporary in nature. Likewise, CAV infections in the first three to five days are profoundly damaging to the immune system, but even late infections can result in a transitory compromise in immune function. Several studies have demonstrated the additive effect of these three viruses when birds have been co-infected.

This challenge study showed that the titer of the *E. coli* inoculum can have a big impact on the incidence of cellulitis (2A vs. 2B in table). This underscores the importance of managing the bacterial loads in the environment when trying to control IP condemnations. Pre-challenging this flock with viruses did not seem to affect the incidence or severity of cellulitis (2A vs. 3A) when a high titer of *E. coli* was given – perhaps because this number of organisms was already overwhelming even the immunologically intact birds. However, the viral pre-challenge did have a significant impact on the incidence of cellulitis when the lower titer of *E. coli* was given (2B vs. 3B). This suggests that the viral pre-challenge was enough to precipitate cellulitis in a flock that otherwise would have been capable of resisting the lower titered inoculum. As one would expect, virally pre-challenged birds receiving the Hi-dose had a higher incidence of cellulitis than the Lo-dose group (3A vs. 3B). It is interesting that this group also had greater weight suppression than the other virus groups, while the *E. coli* only groups had no weight suppression at all.

To summarize, in addition to limiting conditions which lead to skin scratches, this study demonstrates the importance of controlling bacterial loads as well as immunosuppressive viruses in managing infectious process in broilers.

Table 1. Summary of 32 day findings related to each challenge isolate.

Group Information		Reovirus	IBDV	CAV		*E. coli*	
ID#	**Description**	**Body Weights**	**Intact bursa (histo)**	**Intact thymus (histo)**	**PCR results (pos.)**	**Mean lesion score**	**Cellulitis incidence**
1	Negative Controls	1664 a*	100%	92%	NT	0.0 a	0% a
2A	*E. coli* SubQ-Hi dose	1681 a	100%	100%	NT	2.2 b	91% c
2B	*E. coli* SubQ-Lo dose	1632 a	100%	100%	NT	0.0 a	0% a
3A	Viral challenge/ *E. coli* SQ-Hi	1453 b	11%	75%	1/3	2.2 b	75% c
3B	Viral challenge/ *E. coli* SQ-Lo	1548 b	18%	82%	1/4	1.5 b	36% b
4	Viral Challenge Only (Reo/CAV/IBDV)	1545 b	10%	100%	1/4	0.0 a	0% a

* Groups within a column not sharing a same letter are significantly different ($p<0.05$).

RESULTS OF TWO YEARS OF INFLUENZA SURVEILLANCE IN WILD BIRDS AND BACKYARD FLOCKS OF NORTHERN ITALY

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ABSTRACT

Wild waterfowl (*Anseriformes* and *Charadriiformes*) are considered to be the main reservoirs of avian influenza viruses (8, 9) and represent the biological source from which avian influenza (AI) can be introduced to poultry (1).

Following the AI epidemics occurring in Italy between 1997 and 2003 (2, 3, 4), a surveillance program funded by the Italian Ministry of Health was implemented. In the framework of this program, in addition to monitoring in intensively reared poultry, virological surveillance of avian influenza was undertaken in wild birds and backyard poultry flocks in order to investigate their role in the maintenance of AI infection in densely populated poultry area (DPPA).

From December 2003 to January 2006 164 backyard flocks and more than 4,000 wild birds in Italian wetlands were sampled in three regions in the North of Italy (Veneto, Lombardia and Emilia-Romagna).

In backyard flocks a minimum of 10 cloacal swabs from each representative species, and at least one sample of fresh droppings were collected for AI detection. Samples collected from wild birds (cloacal swabs) were screened by means of Real Time RT-PCR (RRT-PCR) (5, 7), then processed for virus isolation in embryonated fowl's SPF eggs according to EU Directive 92/40 (6).

At the end of the study period, 27 low pathogenic avian influenza viruses (AIVs) belonging to 13 different AIV subtype combinations (H1N1, H1N2, H2N2, H3N8, H4N6, H5N3, H7N7, H9N2, H9N8, H10N4, H10N7, H11N2, H11N9) had been isolated from 19 backyard flocks.

Furthermore, 327 out of 4,083 wild birds were found positive for type A avian influenza in RRT-PCR. From these 327 RRT-PCR positive samples, 49 AIVs have been isolated belonging to 15 different AIV subtype combinations (H1N1, H1N3, H3N8, H4N6, H5N1, H5N2, H5N3, H6N2, H7N4, H7N7, H9N2, H10N4, H10N7, H10N8, H11N9) from seven different species. No H5N1 highly pathogenic avian influenza (HPAI) was present in samples collected during this period.

This study highlights that backyard flocks are at high risk of AIVs introduction. Penetration of AIVs into intensively farmed poultry may occur following amplification in the domestic reservoir. It is also possible that through trade or movement of backyard birds infection may expand significantly, thus representing an increased risk for intensively reared birds. This study also confirms the important role of wild waterfowl in the perpetuation of low pathogenicity avian influenza viruses during the winter season in Southern Europe.

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

SITUATION OF INFECTIOUS BURSAL DISEASE VIRUS IN CENTRAL AMERICA

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INTRODUCTION

Broiler production in Central America is increasing more and more each day. One of the principal problems for the poultry industry is the challenge caused by the infectious bursal disease virus (IBDV), which affects dramatically the performance of birds causing economical losses. In order to keep a good and healthy immune system, vaccination programs have been formulated along with good biosecurity programs.

The infectious bursal disease virus (IBDV) is known for causing immune suppression to birds, especially to young ones. If the disease affects a bird during its first 21 days of age, it will cause permanent immune suppression. The virus is manifested as a clinical and subclinical form of the disease. However, there has been another type of manifestation of the virus, which is the very virulent form that may cause the death of birds. The purpose of this study is to isolate the different variants and strains of the IBDV found in Central America by doing chronological studies using image processing and PCR.

MATERIALS AND METHODS

Samples of bursas were removed from broilers at 21, 28, and 35 days of age from all the countries of Central America. They were measured using the bursameter, and in some cases, spleens were removed for comparison with the bursas. Thymuses were also removed for evaluation. Productive parameters were recorded so they could be compared with the results. Half of each bursa was immersed in 10% formalin and the other half on FTA Cards for PCR analysis. The ones in formalin were used for image processing. Samples were sent to the U.S. for histopathology and PCR analysis (to Mississippi Veterinary Diagnostic Laboratory and to Ohio State University respectively).

RESULTS

According to PCR results from the Ohio State Laboratory, some IBD viruses have been found belonging to the molecular groups 2, 3, 5, and 6 from Dr. Jackwood's PCR reference chart. There were others that showed new patterns that haven't been classified yet. (Table 1)

Clinical cases of IBDV involving mortality have been found in Guatemala, El Salvador, Costa Rica, Nicaragua, and Panama. There is an investigation now on determining the very virulent virus of IBD, due to clinical signs and manifestations shown in birds in El Salvador and Guatemala.

Table 1. Classification of IBDV according to its molecular group and new patterns found in Central America.

Country	Molecular Group	New Patterns
Guatemala	2 (Delaware E)	
	5	BstN1 424 172 119 MbO1 480 229
Costa Rica	6 (variant)	BstN1 424 172 119 MbO1 362 229
	3	
Panama	6	BstN1 215 209 172 119 MbO1 362 229
		BstN1 424 172 119 MbO1 362 270 112
		BstN1 424 410 215 209 172 119
		MbO1 362 270 229

***** More data regarding El Salvador, Nicaragua, Guatemala, Costa Rica, and Panama will be presented at the WPDC.

HEPATIC LIPIDOSIS IN TURKEYS: BEYOND THE CURRENT PATHOGENETIC HYPOTHESIS

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ABSTRACT

Hepatic lipidosis (HL) is a condition described in young turkey hens and characterized by enlarged livers mottled by pale yellow areas. Histologically, the main feature is fatty degeneration associated with necrosis and hemorrhage. The cause of HL is uncertain although nutritional and metabolic factors are suspected. Over a period of two years, we observed five cases of a hepatopathy with gross and microscopic changes consistent with HL. Two of these cases affected turkey breeder hens of 12 and 21 weeks. Interestingly, another case was observed at the slaughterhouse in 20 weeks commercial male turkeys, whereas two cases involved commercial poults of 8 and 26 days. A histochemical approach could better detail this condition and help speculating about its pathogenesis.

INTRODUCTION

Some hepatic disorders characterized by fatty change can affect avian species: e.g. fatty liver hemorrhagic syndrome and fatty liver and kidney syndrome are widely investigated hepatoses affecting chickens (7, 3, 1). On the contrary, hepatic lipidosis (HL) of turkeys has been surprisingly neglected by pathologists so far with only one report in the literature (2). In particular, morphopathological comparison between HL and aforementioned avian hepatoses is still lacking. Moreover, hepatic changes consistent with HL occurred recently in turkeys at an unusual age (less then one month or at slaughter age) but with the typical transient increase in mortality we know in the hens.

Finally, the possible copper over-supplementation in turkey diets suggested that we consider another hepatopathy described in mammals and related to copper storage disorder in the liver: Wilson's disease, whose features include liver fatty change (8).

MATERIALS AND METHODS

Cases. Over a period of two years liver samples or dead birds from five cases of suspected HL were submitted to our laboratory for diagnosis. Case 1 and 2 were from flocks of breeder hens of 12 and 21 weeks of age respectively with transitory increased mortality. Case 3 was from male commercial turkeys of 144 days. Only samples of affected livers were collected at the slaughterhouse, although heart changes were also observed. No further information about the flock was provided. Case 4 was from commercial female poults of 26 days that showed a doubled daily mortality for four days (0.1%). Case 5 was from eight-day-old male poults. In this flock the increase in mortality was also referable to recent debeaking and to rotaviral enteritis confirmed by electronmicroscopy.

Histopathology. Formalin fixed, paraffin embedded liver samples were cut at four microns and stained with hematoxylin-eosin for routine microscopic examination, with James's stain for reticulin fibers (4), and with rhodanine stain for copper (5).

RESULTS

Grossly, we could evaluate poults only from case 4. These animals were in good condition. The livers appeared enlarged and mottled by pale yellow areas. No other lesions were observed. From case 3 we could observe entire formalin fixed liver lobi and from case 5 the whole liver. Both showed similar mottled aspect with yellowish areas intermingling with large hemorrhages.

Microscopic features observed in young breeder hens from cases 1 and 2 overlapped the findings observed in cases 3 and 4 from commercial turkeys.

The association between hepatocyte fatty change and hemorrhages was constant. The latter were medium size to large and predominantly centered on central veins. The fatty change was characterized by large intracytoplasmic vacuoles distorting the hepatic cords. Mild biliary hyperplasia and a few scattered lymphocytes were also detected. In case 5, the fatty change was mild to moderate with diffuse hemorrhages and random hepatocellular necrosis being the main features. In all cases the reticular framework was thoroughly maintained as confirmed by James's stain. Rhodanine stain excluded the presence of copper-containing granules in liver sections of the five cases.

DISCUSSION

Fatty change and hemorrhages consistent with HL can occur not only in turkey breeder hens but also in commercial turkeys (both male and female). In the latter case, low protein diets cannot play a role in the pathogenesis of HL as hypothesized for breeder hens (2) since such dietary restrictions are obviously unnecessary in commercial turkeys. Liver fatty change can be related neither to excessive dietary fat nor to massive delivery of free fatty acids from adipose tissue which is minimal till slaughter age in this species. The involvement of copper metabolism disorders was also excluded.

A possible pathogenesis, if any, unifying all the cases we observed could be found in some transient injury of the hepatocyte leading to lipid accumulation in the cell.

The integrity of reticulin framework in HL differentiates this condition from similar hepatopathies as fatty liver hemorrhagic syndrome and fatty liver and kidney syndrome (7) in which liver rupture, possibly related with reticulolysis, is common. We can speculate that the maintenance of the reticulum, which is necessary for liver regeneration (6), explains the transient mortality in HL affected flocks and subsequent recovery with normal productive performances.

(The full-length paper will be submitted for publication in a journal.)

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EFFECT OF YEAST CELL WALL ON NEWCASTLE AND COCCIDIA VACCINATION IN BROILER DIETS

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SUMMARY

Yeast cell wall was tested as a possible immunomodulator on vaccination process in broilers. One hundred and twenty chicks were used and divided into four different groups, each with a different diet: 1 = control, 2 = control + yeast cell wall (YWC), 3 = control + flavomycin, and 4 = control + YWC + flavomycin. Another experiment was carried out to evaluate the immune response against coccidia. Three diets were used in three groups: 1 = control, 2 = control + PCL, and 3 = PCL and a commercial vaccine used as an immunogenic stimulus at one day post hatching.

Cell mediated immune response. Interdigital membrane of the feet was used as an indicator of the classical basophilic cell hypersensitivity as *in vivo* lymphoproliferative response. The PHA-P mediated swelling was significantly different ($p \leq 0.001$) in the YWC treatment compared with other diets.

Humoral response. Humoral immune response was evaluated with serum at 14 days against Newcastle disease. Broilers were immunized previously in the groups with YWC, antibiotic, and the combination. The groups treated with YWC had a better humoral immune response against Newcastle disease vaccine. (Figure 1)

The immune response against *Eimeria* showed a significant difference ($p \leq 0.001$) in the YWC treatments compared with the one without YWC supplementation.

Dietary supplementation with immunmoludators such as yeast cell wall have been a point of attention. The literature has reported many differences with the utilization of these immunmoludators. The results of these experiments have demonstrated the beneficial effect on the vaccination process on broilers, but the most important thing is trying to explain the molecular way how they work and the appropriate conditions for its function.

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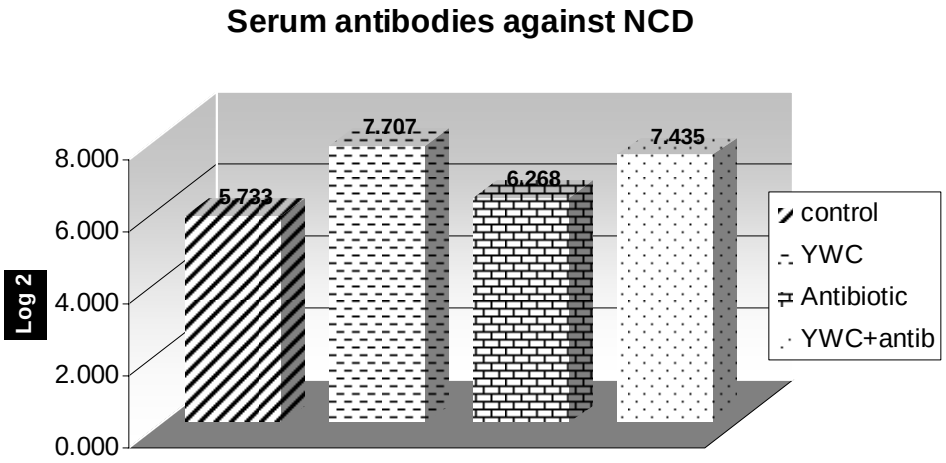
Figure 1. Total serum antibodies against Newcastle disease (NCD) with HI technique.



ANTIBODY DETECTION AGAINST RETICULOENDOTHELIOSIS VIRUS IN LAYER HEN FARMS USING ELISA TEST

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SUMMARY

The present study was conducted to find out the frequency of antibodies against the reticulo-endotheliosis virus (REV) in layer hen from commercial farms using the ELISA test. A total of 630 serum samples were collected in 42 flocks of 20 farms of layer fowls located in the region of Lima and of La Libertad of Peru. The results showed that 33.3% (14/42) of flocks were seropositive and among them, 22.2% (6/27) were from the Lima region and 53.3% (8/15) from La Libertad, indicating the presence of the virus in more than 7% of the population, which was the prevalence reference value. No statistical association was found between the three variables under evaluation (history of tumor problems in the flock, genetic lines, and age) with the results of the diagnostic test. This is the first study conducted in commercial layer fowls showing the presence of the REV in Peru. Serosurveillance monitoring in various regions of the country is highly recommended, including other commercial avian species to identify the current status of this virus in the country, and to establish suitable health controls.

RESUMEN

El estudio tuvo como objetivo la detección de anticuerpos contra la infección con el virus de la reticuloendoteliosis (REV) en gallinas ponedoras comerciales mediante la prueba de ELISA. Entre julio del 2004 y marzo del 2005 se obtuvieron 630 muestras de suero de 42 lotes en producción de 20 granjas de gallinas ponedoras comerciales localizadas en la provincia de Lima y el departamento de La Libertad, Perú. Se encontró el 33.3% (14/42) de lotes seroreactores; de estos, 22.2% (6/27) fueron de la zona de Lima y 53.3% (8/15) de La Libertad, demostrando la presencia del virus en más del 7% de la población estudiada que fue la prevalencia referencial utilizada. No se encontró una asociación significativa en ninguna de las tres variables evaluadas (antecedentes de problemas tumorales, grupo etáreo y línea genética) con los resultados de la prueba diagnóstica. Este es un primer estudio realizado en gallinas ponedoras cuyos resultados evidencian la presencia de la REV en el Perú. Se recomienda realizar monitoreos serológicos en diferentes zonas geográficas del país, incluyendo otras

especies aviares de crianza industrial, para definir la situación de la presencia del virus en el país, y establecer la vigilancia y control sanitario adecuado.

INTRODUCTION

The reticuloendotheliosis virus (REV) has a wide variety of hosts, even more than other avian tumor viruses (3, 8), and its distribution in several countries has been larger than expected (3). REV virus infection has been detected in high proportion of layer hens and broiler fowls and turkey flocks in USA (4).

REV together with Marek's disease and avian leukosis are considered important pathogens from the economic viewpoint by the World Organization for Animal Health (OIE) (5). This disease causes economic losses due to mortality caused by tumors and immune suppression as well as export offspring ban of infected flocks. Also, REV is a potential contaminant agent of live virus vaccines, so vaccine producer companies and specific-pathogen-free (SPF) farms keep a tight serosurveillance of this virus in their products (1, 10).

Enzyme-linked immunosorbent assay (ELISA) commercial kits are currently available. This is a quick, effective, sensitive, and useful enzyme immunoassay for the detection of antibodies in positive birds, and to confirm the absence of virus in pathogen-free flocks and in flocks producing offspring for exportation (10).

In a previous study conducted in Peru (6) in 12 flocks of layer breeders and broilers, older than 50 weeks of age, was shown that all were negative to the presence of REV antibodies; however, the possible presence of this virus in layer fowls was unclear. For this reason, the present survey was carried out to detect the presence of antibodies against REV virus in flocks of layer fowls in the province of Lima and the department of La Libertad, using an ELISA commercial test.

MATERIALS AND METHODS

Sample collection. Sera were obtained from July 2004 till March 2005 in 28 commercial hen layer farms located in the province of Lima and in the northern department of La Libertad. The minimum number of samples was determined by the formula for the detection of diseases in a population. A total of 630 serum samples were collected from 42 flocks (15 birds per flock). Fowls were classified according to three variables: history of tumor problems in the flock (presence or absence), genetic lines (Hy Line, Isa Brown, Lohman, and Brown Link), and age (20-35, 36-50, 51-65, and >65 weeks of age) weeks old, 40% (4/10) in flocks of 36 to 50 weeks old, 12.5% (2/16) in flocks of 51 to 65 weeks old, and 50% (4/8) in flocks older than 65 weeks, The collected sera were processed in the Laboratory of Avian Pathology School of Veterinary Medicine San Marcos University.

Antibody assay. Sera were analyzed by the ELISA test for antibody detection using the IDEXX commercial kit. Samples were diluted at 1:500 in a buffer solution following the instructions indicated by the manufacturer laboratory. Optical densities (OD) were measured at 650 nm using an ELISA ELX 800 Reader (Biotek Intruments, Inc). Serum samples with an M/P $\leq$ 0.05 were considered negative and those with >0.5 were considered positive.

Statistical analysis. Results were expressed as percentage with the respective 95% confidence interval. A flock was positive if at least one serum resulted positive. Data were recorded in a database and chi-square test was used to evaluate the association of positive flocks with the three variables under evaluation.

RESULTS AND DISCUSSION

REV antibodies were detected in 33.3 ±14.2% (14/42) of flocks during the production period. The detected level was higher than the expected 7% reference value. 22.2% (6/27) of flocks from the Lima region were seropositive whereas 53.3% (8/15) from La Libertad region were seropositive (Table 1) indicating the presence of REV in layer hens in the studied zones. Data from a previous study in breeder farms (6) could only reveal three positive sera out of 180, but OD values were lower than the positive controls; however, in this study, some of the positive flocks showed high frequency of infected birds and moreover, high OD values which may indicate that these birds are in fact true positives.

Vertical transmission of the disease might be disregarded due to previous studies in the area (6). On the other hand, the risk of infection is higher in commercial farms than in layer breeder farms due to the relative lower biosecurity measures. This may explain the greater level of infection obtained in the present study.

Comparative results were found in commercial farms in USA (12), where the frequency of infection was 21% in flocks without history of tumor problems. In the present study, some flocks reported an increased frequency of tumors since lymphoid chronic neoplasia and runting disease syndrome are common clinical signs caused by REV (7). The frequency of seropositive REV flocks with history of tumor problems was 38.6% (5/13) in comparison with 31.0% (9/29) in flocks without such a history; however, statistical differences were not observed. These

findings may indicate that the occurrence of tumors in seropositive flocks are not necessary caused by REV as other pathogens such as avian leukosis virus and Marek's disease virus.

Nowadays, clinical signs in chickens are mainly associated to contaminated vaccines with REV (8). The most likely route of infection in this study was horizontal; nevertheless, the use of contaminated vaccines would be a possibility, especially in flocks with history of tumors. Natural reservoirs could be another source of infection (9), especially when considering that frequency of REV is higher in temperate and subtropical regions due to a greater presence of wild birds (13).

In relation to age groups, frequency of REV was 50 (4/8), 40 (4/10), 12.5 (2/16), and 50% (4/8) in flocks of 20-35, 36-50, 51-65, and >65 weeks of age), but without statistical differences. These results were similar to others (11) showing that antibodies can be related to age and may persist for different periods. There is a report pointing out that antibodies in infected turkeys remained in large number of birds up to 40 weeks (2).

The genetic line was analyzed in relation to frequency of REV infection. Results indicated that 45.5% (10/22) of the Hy Line flocks, 28.6% (2/7) of the Isa Brown flocks, 9.1% (1/11) of the Lohman flocks, and 50% (1/2) of Brown Link flocks were seropositive, but without significant difference between genetic lines.

Based on the results and on the increasing prevalence in other countries, frequent serologic monitoring should be considered to evaluate the dynamics of the disease in bird flocks, both in layer fowls and other commercial avian species. It is important to include reticuloendotheliosis as part of the differential diagnosis when tumors are found in layer hens. Likewise, epidemiologic serosurveillance strategies should be established to avoid possible economic losses to the Peruvian poultry industry.

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Table 1. Frequency of reticuloendotheliosis virus in layer fowls from commercial farms located in Lima and La Libertad regions of Peru (2004-2005).

Region	Flocks	
	N° positives / N° examined	Frequency (%)
Lima	6/27	22.2
La Libertad	8/15	53.3
Total	14/42	33.3 ± 14.2

PARASITIC PROVENTRICULITIS IN THE HOODED CROW (*CORVUS CORONE CORNIX*)

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SUMMARY

During a health monitoring program of the crow (*Corvus corone cornix)* carried out in Northern Italy in 2003-2006, two different nematodes were observed in the proventriculus of 44 out of 276 examined birds. Clinical evaluation, necropsy, histology, and morphological identification of the parasites were performed. All affected birds were in good condition and no clinical signs were detectable. Thirty-eight (13.8%) crows were infected with *Dispharynx nasuta* and six (2.2%) with *Tetrameres fissispina*. This is the first report of *D. nasuta* and *T. fissispina* in the hooded crow.

INTRODUCTION

The hooded crow (*Corvus corone cornix*) is widely distributed throughout Italy and its increasing presence is related to its role of scavenger both in urban and rural areas. During a study primary carried out in Northern Italy from October 2003 to October 2006 to investigate the potential role of the crow as reservoir of zoonotic pathogens we found a relatively high incidence of stomach worms (*Dispharynx nasuta* and *Tetrameres fissispina*) in the proventriculus of the hooded crow.

MATERIALS AND METHODS

Over the three-year study period, 276 crows (juvenile and adult) were captured and euthanized after the clinical examination. Gross examination was performed and each proventriculus was opened longitudinally and examined for the presence of nematodes. All worms were collected, fixed in 70% alcohol, cleared in lactophenol, and examined under a stereomicroscope. The helminths were identified according to Norton and Ruff (2). Samples of *T. fissispina*-infected proventriculi were collected and fixed in 10% buffered formalin for histological examination. Paraffin embedded sections were cut at 4 μm and stained with hematoxylin-eosin.

RESULTS

All the examined crows showed no clinical signs and were in good body condition. Grossly, 38 birds showed dilated proventriculus and the proventricular mucosa was covered by numerous white filiform nematodes. Six crows had 1-2 mm in diameter red-stained nodules scattered throughout the proventricular wall and visible through the serosal surface. Based on the morphological features evaluated under the stereomicroscope *D. nasuta* was identified in the 38 (13.8%) crows with dilated proventriculus and *T. fissispina* in the six (2.2%) birds with the red-stained nodules. Males and females of *T. fissispina* were easily identified due to their marked sexual dimorphism. In

the crows infected with *T. fissispina*, occasional intraductal adult nematodes were histologically detectable. The tubular glands were markedly dilated by adult female nematodes with an enlarged uterus containing numerous 30 µm x 50 µm, oval, thick-shelled, embryonated eggs. The lamina propria was only occasionally mildly infiltrated by small numbers of lymphocytes.

DISCUSSION

D. nasuta and *T. fissispina* are nematodes of the order *Spirurida* and their life cycle is indirect with pillbugs, sowbugs or other isopods as intermediate hosts for *D. nasuta* and amphipods, grasshoppers, earthworms, and cockroaches for *T. fissispina*. *D. nasuta* has been reported in chicken, turkey, grouse, guinea fowl, partridge, pheasant, pigeon, quail, and a number of passerine birds in North and South America, Africa, and Asia. The adult worm is located in the proventriculus and esophagus, rarely in the small intestine (1, 2, 3). *T. fissispina* is a common parasite in chicken, turkey, duck, guinea fowl, goose, and pigeon worldwide. The adult worm is found in the proventriculus where the females are embedded deep in the glands and are easily seen from the serosal surface as dark red spots (1, 2, 3). Both nematodes may be responsible for proventriculitis leading to a debilitating status characterized by emaciation and anemic conditions. Our results suggest that these nematodes have an overall low pathogenic potential in the hooded crow, irrespective of the large numbers of the parasites. In our study all the infected crows were in good condition and the proventriculus was only minimally to mildly affected. *D. nasuta* and *T. fissispina* are reported to infect a broad range of natural hosts (common crow included), but interestingly this is the first report of *D. nasuta* and *T. fissispina* infection in the hooded crow.

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EXPERIMENTAL INDUCTION OF AMYLOIDOSIS IN CHICKENS

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Amyloidosis is a group of diseases characterized by extracellular deposition of protein that contains non-branching, straight fibrils on electron microscopy that have a high content of ß-pleated sheet conformation (2, 4). In humans, at least 24 biochemically distinct proteins can transform into amyloid fibrils. In birds, spontaneous cases of amyloidosis are usually of the reactive or AA-type (2). AA amyloidosis is frequently associated with chronic inflammatory disease. The precursor of AA is acute phase serum amyloid A (SAA) protein and mainly originates in the liver (2, 4). The concentration of SAA in the blood increases within 24 to 48 hrs after trauma, infection, or inflammation. The synthesis and secretion of SAA is mediated by pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor-α (2).

Rapin *et al*. (3) described an outbreak of systemic amyloidosis with moderate to high mortality in two young layer flocks after repeated vaccinations with oil-emulsified bacterins. The amyloid deposits were present in the liver, spleen, and kidney, and frequently accompanied by hepatic ruptures and hemorrhages. Amyloid arthropathy with systemic amyloidosis has been described as a clinical problem associated with growth depression and lameness in commercial brown layers (2). Massive accumulation of amyloid protein occurs in and affects the superficial articular cartilage, the nutritional vessels and the synovium. Landman *et al*. (2) isolated *Enterococcus faecalis* from a case of joint amyloidosis. Induction of amyloidosis has been described in chickens using serial injections of living Staphylococcus aureus cultures. Druet and Janigan (1) used injections of sulfanile azocasein to produce

amyloid in hens. Wang and Di (5) reported liver amyloidosis can also be induced by repeated injections of *Salmonella pullorum*. In the present study, amyloidosis was investigated in chickens experimentally inoculated with mixtures of killed bacteria and adjuvant.

MATERIALS AND METHODS

Twenty-six 28-day-old specific pathogen free chickens were used. The chickens were divided into a treated group (n = 21) and a control group (n = 5). They were fed *ad libitum* with a commercial diet. To induce amyloidosis, a high concentration of formalin-killed *Salmonella enteritidis* was suspended in phosphate buffered saline (PBS, pH 7.2) at a concentration of 4.8 × 1010 cells/mL and emulsified with an equal volume of mineral oil adjuvant. At 28 days of age, the treated group received intramuscular injections in the left leg 2.0 mL of the emulsions and 1.0 mL (corresponding to a double dose) of each of two commercially vaccines made by Nisseiken (Ohme, Japan). One was coryza vaccine (types A and C) in aluminum salt and the other was *Mycoplasma gallisepticum* vaccine in aluminum salt. These three inoculations were repeated three weeks later in the right breast and six weeks later in the left breast. Five of the 21 chickens died between 26 to 49 days after the first inoculation and the survivals were necropsied at 21 days after the third inoculation. Control chickens remained untreated through the experiment.

The liver, spleen, kidney, heart, lung, thymus, bursa of Fabricius, proventriculus, and muscles were removed and fixed with 4% paraformaldehyde solution. All tissue samples were then embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE) and Congo red. Congo red-stained slides were viewed under polarized light for detection of the characteristic green birefringence of amyloid while other slides were examined under light microscopy. Formalin-fixed, paraffin-embedded tissues of livers and muscles were examined for the presence of chicken SAA or IL-6 mRNA by *in situ* hybridization (ISH).

Heparinized blood of both groups was collected from the brachial vein once per week. Serum SAA concentration was determined by a commercial enzyme-linked immunosorbent assay kit (TP-802M; Tridelta, Maynooth Co. Kildare, Ireland).

RESULTS

Clinical signs were noticed in all the chickens of the treated group. Chickens showed lameness in varied degrees from 1 to 18 days after the first inoculation. The mortality rate was 23.8% (5/21) in the treated group. Gross lesions were mainly present in the liver and the muscles at the injection sites. The livers appeared firm, markedly enlarged, and the capsular surfaces were smooth. Hepatic parenchyma showed inconsistent discoloration with patchy green to yellowish-brown areas or sometimes red-brown areas. These findings were seen in 33% (7/21) of the treated group. The injection site in all birds showed moderate to severe swellings, edema, and yellowish-white discoloration of skeletal muscles. The other visceral organs were not visibly affected.

Microscopically, amyloid substances accumulated in Disse's space of the liver, in vascular walls of arterioles in the white pulp of the spleen, and in glomeruli of the kidney. These substances stained positively with Congo red. In Congo red-stained sections amyloid deposits showed apple green birefringence when viewed with polarized light. Ultrastructurally, the amyloid deposits consisted of components arranged in irregular arrays of parallel or crossed fibrils. Individual fibrils were of similar diameter (7 to 10 nm) but varied in length. The incidence of amyloidosis was 66.7% (14/21). SAA mRNA was detected by ISH in the hepatocytes of severely affected chickens at high levels whereas sections pretreated with RNase were negative. The muscles at the injection sites had moderate to severe inflammatory changes with infiltration of macrophages, lymphocytes, and rarely multinuclear giant cells. Various sized cysts containing amorphous eosinophilic and inflammatory debris were evident in the lesions. Lymphocytes, plasma cells, and heterophils were scattered among the lesions and surrounding skeletal muscles. Fibroblasts with collagen were numerous in the outermost layers. IL-6 mRNA was detected by ISH in the macrophages in and around the lesions. Expression levels of IL-6 mRNA and serological SAA levels were higher in the severely affected chickens than in the controls.

DISCUSSION

In the present study, amyloidosis was demonstrated by Congo red stain and electron microscopy. In addition, expression of SAA mRNA in the hepatocytes and increased levels of serological SAA were confirmed in severely affected chickens. High expression of IL-6 mRNA in the injection sites may be related to an increase in the production of acute phase proteins in the liver of severely affected chickens. The results suggest that injecting mixtures of killed bacteria and adjuvant can induce AA amyloidosis in chickens.

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A Δ*tia* MUTANT OF AN AVIAN PATHOGENIC *ESCHERICHIA COLI*

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ABSTRACT

Avian colibacillosis, which is caused by avian pathogenic *Escherichia coli* (APEC), is responsible for large financial losses to the poultry industry worldwide. A better understanding of virulence mechanisms of APEC is needed to guide development of specific disease control methods. Recently, our laboratory described a novel pathogenicity island (PAI) of an APEC O1:K1:H7 strain (APEC O1) that we termed $PAI1_{APEC\text{-}O1}$. Among other genes, this PAI contains a gene which has 94% nucleotide and amino acid sequence homologies to the *tia* locus of enterotoxigenic *E. coli* (ETEC). In ETEC, the Tia protein induces adherence and invasion of epithelial cells. This gene also shows significant homology with the *hraI* agglutinin gene of porcine ETEC and limited homology with the *Yersinia ail* locus. We constructed an isogenic mutant of APEC O1 by knocking out the *tia* gene to see whether this gene is involved in APEC pathogenicity. The virulence of this mutant was tested by subcutaneous and intra-tracheal challenge of one-day-old chicks and aerosol challenge of two-week-old chickens. The *tia* APEC O1 mutant was not attenuated as compared to the wild-type parent strain and was equally able to colonize the lungs and tracheas of infected chickens irrespective of the route of challenge. Taken altogether, these results demonstrate that the *tia* gene does not play a significant role in APEC O1s pathogenesis.

(The full-length article will be published in *Infection and Immunity*.)

EFFECTS OF POULTRY LITTER TREATMENT (PLT) AND ALUMINUM SULFATE (ALUM) ON AMMONIA AND BACTERIAL LEVELS IN POULTRY LITTER

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SUMMARY

Chemical litter treatments are commonly used to reduce ammonia and bacterial levels in poultry litter. They typically accomplish this by acidifying the litter. By acidifying the litter, the production of ammonia and growth of bacteria is inhibited. Two commercially available litter treatments that are commonly used are PLT and Alum. In order to test their efficacy, two experiments were performed on fresh pine shavings in which these litter treatments were added at 22.7, 45.5, and 68.2 kg per 92.9 m^2. Broiler chickens were raised on this litter under typical commercial conditions for seven weeks. The following measurements were taken weekly: ammonia, pH, percent moisture, and bacterial levels (total aerobic, total anaerobic, *C. perfringens*, and *Staphylococcus* in CFU/g). There was a decrease in pH and ammonia levels in all of the treated litter; however, there was no difference in bacterial levels or moisture content for any of the treatments.

INTRODUCTION

During the typical life span of commercial broilers, one metric ton of litter is produced per 1,000 birds. Over the course of a year this could lead to 120 metric tons of litter produced in one 20,000 bird poultry house. This built-up litter can result in high ammonia levels, which can adversely affect poultry and human health. Additionally, high ammonia levels make the birds more susceptible to respiratory diseases, like ILTV or airsacculitis. Methods to reduce ammonia levels and pathogenic microbes include changes in management practices and litter treatments. Though these methods work, they are not without problems. One example is litter treatments, which are typically effectual for three to four weeks before they lose effectiveness. The problem is that depending upon the target market, chickens are typically housed for 6+ weeks. The goal for this study was to determine the optimal application rate for prolonging the effectiveness of litter treatments in reducing both ammonia and bacteria. Two experiments were performed in which PLT and Alum was used at differing application rates.

MATERIALS AND METHODS

Trial 1

Housing. Clean pine shavings were placed to a depth of 8 cm in 16 environmental chambers (2.44 x 2.44 x 2.44 m). One-day old chicks were acquired from a commercial hatchery and 70 were randomly placed into each chamber.

Litter treatments. Poultry Litter Treatment (PLT) was applied according to manufacturer's instructions, 22.7 kg per 92.9 m^2. Two additional levels were also utilized 45.5 and 68.2 per 92.9 m^2 to see if the additional product would significantly affect the results. In addition to the three treated groups there was a non-treated control group. Each treatment group consisted of four randomly assigned chambers.

Ammonia measurements. The Drager CMS Analyzer equipped with the remote air sampling pump was used using the appropriate ammonia CMS chip (0.2-5ppm, 2-50ppm and 10-150ppm). The tube from the sampling pump was inserted into a (36 x 46 x 12 cm) container and then run for 60 seconds. If after 60 seconds there was no reading, additional time would be given (up to 300 seconds).

Litter collection. Litter was collected weekly, starting the day prior to chick placement and continued for seven weeks. Collection was performed in each pen by using the grab sampling technique (1). In brief, clean-new gloves are used to collect samples from three areas within each pen. This included litter from under the nipple drinkers, next to the feed troughs and the middle of the pen. The three collected samples were than thoroughly mixed together in a sterile stomacher bag and transported back to the lab for analysis.

Microbiology. Total aerobic, total anaerobic, *C. perfringens* and *Staphylococcus* levels were enumerated (CFU/g) for each pen. This was performed using plate count agar (PCA), reduced blood agar (RBA), tryptose sulfite cycloserine agar (TSC), and *Staphylococcus* medium 110 (m110) respectively. Dilutions were performed by adding 10 g of litter to 90 mL of sterile physiological saline (0.75% NaCl). This produced a 10^{-1} dilution. Further dilutions were performed by transferring 10 mL into another 90 mL

sterile saline bottle; this was performed until dilutions ranging from 10^{-1} to 10^{-8} were made. The dilutions were then spiral plated in duplicate onto their respective media types and incubated under appropriate conditions. PCA and m110 were incubated aerobically at 37°C; while RBA and TSC were incubated at 37°C in an anaerobic chamber containing 5% CO_2, 5% H_2, and 90% N_2. After 18 hours colonies were quantified on a digital plate reader and average bacterial counts obtained.

Determination of pH. Litter samples were mixed 1:1 with distilled water and allowed to sit overnight at 4°C. The following day pH was measured in triplicate and recorded.

Percent moisture. After litter arrival in the lab, 10g of litter was weighed and placed in a drying oven that was set for 150°C and allowed to dry for 36 hours. After 36 hours the samples were reweighed and percent moisture was determined.

Statistical analysis. All of the data was analyzed using SPSS version 12.0 using GLM procedure. If there was a significant difference ($P \leq 0.05$), means would be analyzed using Tukey's Multiple Comparison Test. Before analysis all percentage data was arcsine transformed to normalize this data. Additionally, CFU/g counts were normalized for analysis by using log 10 transformations.

Trial 2

The same methods were used in trial 1, except aluminum sulfate (Alum) was used in place of PLT.

RESULTS AND DISCUSSION

The pH results for PLT are presented in Table 1. After two weeks the acidic effects of PLT applied at the rate of 22.7 kg have diminished so that its pH was the same as the untreated group. Both the 45.5 and 68.2 kg application rates kept the pH lower then untreated and 22.7 kg rate until week seven.

Ammonia levels were the same until week five, at which time all treated groups had significantly lower ammonia levels then the untreated group (Table 3). By week six, only the 68.2 kg group had significantly lower ammonia levels then the untreated group. By the seventh week there was no difference in ammonia levels between the four groups.

Bacteriologically there was no difference detected between the four groups. The only difference ($P \leq 0.05$) detected was with the 68.2 kg group on week seven. During that week this group had lower anaerobic bacterial numbers (11.8) then the untreated group (12.4). There was no difference between the four groups when litter moisture was measured.

Alum treated litter produced a variable pH among the treatments (Table 2). Generally though, the groups that contained the highest levels of Alum maintained a lower pH ($P<0.05$) over six weeks. Though the pH for Alum wasn't as low as that for PLT treated groups, they both had similar buffering capabilities. The three application rates (22.7, 45.5 and 68.2 kg) for both PLT and Alum lost their buffering capabilities during the same weeks (weeks two, four, and seven). Similar pH and buffering capacity results were observed by Line and Bailey (2) when they applied 3.63 kg per 9.3 m^2 (36.6kg/93m^2) with both sodium bisulfate (PLT) and aluminum sulfate (Alum).

Ammonia levels for Alum (Table 4) were higher then what was observed for PLT treated groups. Before week six the three treated groups had lower ammonia levels then the control; however by week six there was no difference in ammonia levels between the four groups.

Bacteriologically the only difference between the four groups was observed at week seven, with the 68.2 kg Alum rate having lower anaerobic bacterial numbers (9.7) then untreated litter (11.1) and the 45.5 kg treatment (11.4) with a $P<0.05$. Moisture levels were unaffected by the four groups for the duration of the trial. This lack of differences in moisture level when using Alum differs than what was reported recently (3). This difference could be due to our use of pine shavings, while in that trial they used clean rice bran and had an application rate of 1.15 kg /m^2 compared to our highest level of 0.73 kg/m^2.

From these results it was concluded that neither PLT nor Alum affect either bacterial numbers or litter moisture levels significantly in fresh pine shaving litter. Perhaps the observation in reduced anaerobic numbers on week seven with the highest application rate implies that long term usage of either of these products may reduce some bacterial populations. Additionally, the low initial ammonia levels are due to the use of clean litter. Subsequent research will be performed in which long term usage of these litter treatments will be.

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Table 1. The pH levels associated with PLT treated bedding. PLT was applied at three levels 22.7, 45.5 and 68.2 kg per 92.9 m^2 before chick placement and measurements were taken weekly. Letter differences signify statistically significant differences at $P \leq 0.05$.

PLT	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Untreated	6.35[a]	6.30[a]	6.52[a]	6.82[a]	6.67[a]	7.95[a]	8.47[a]	8.75
T22.7	2.45[b]	2.52[b]	5.75[a]	6.52[a]	6.57[a]	7.82[a]	8.12[a]	9.25
T45.5	2.32[b]	2.42[b]	4.47[b]	5.40[b]	6.40[a]	7.27[b]	7.47[b]	8.72
T68.2	2.28[b]	2.32[b]	4.07[b]	5.02[b]	5.57[b]	6.95[b]	7.32[b]	9.03

Table 2. The pH levels associated with Alum applied at three levels 22.7, 45.5 and 68.2 kg per 92.9 m^2 to fresh pine shavings. Letter differences signify statistically significant differences at $P \leq 0.05$.

Alum	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Untreated	6.78[a]	6.35[a]	6.88[a]	6.98[a]	8.58[a]	9.00[a]	9.67[a]	9.55[a,b]
T22.7	4.95[b]	5.20[b]	6.35[a]	6.78[a]	8.40[a]	8.35[a,b]	9.50[a,b]	9.52[b,c]
T45.5	4.38[c]	4.58[c]	5.48[b]	5.55[b]	8.43[a]	8.22[a,b]	9.40[b,c]	9.60[a]
T68.2	3.80[d]	4.12[c]	5.15[b]	5.42[b]	7.65[b]	7.83[b]	9.22[c]	9.40[c]

Table 3. The weekly ammonia levels in ppm associated with PLT treated litter. PLT was applied before chick placement at three levels 22.7, 45.5 and 68.2 kg per 92.9 m^2 to fresh pine shavings. Letter differences signify statistically significant differences at $P \leq 0.05$.

PLT	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Untreated	0	0	0	0	3.18	36.70[a]	69.50[a]	75.00
T22.7	0	0	0	0	0	23.45[b]	47.00[a,b]	64.00
T45.5	0	0	0	0	2.35	17.35[b]	38.90[a,b]	70.75
T68.2	0	0	0	0	0	13.80[b]	26.07[b]	85.67

Table 4. Ammonia levels, in ppm, from pine shaving litter that was initially treated with Alum. Alum was applied at three levels 22.7, 45.5 and 68.2 kg per 92.9 m^2 before chick placement onto fresh pine shavings. Letter differences signify statistically significant differences at $P \leq 0.05$.

Alum	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Untreated	0	0	0	2.83	22.95[a]	77.25[a]	74.00	123.25
T22.7	0	0	0	0.73	14.30[b]	72.75[a]	79.25	121.75
T45.5	0	0	0	0.60	15.57[a,b]	34.17[b]	66.75	135.00
T68.2	0	0	0	0.58	14.85[b]	29.57[b]	68.00	127.75

COMPARISON OF COMMERCIAL AND SPECIFIC PATHOGEN FREE EMBRYONATED CHICKEN EGGS TO TITER POULTRY VACCINES

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INTRODUCTION

Embryonated chicken eggs have been used for biological suspension titration for a long time; however, there is little bibliography about the comparison between both commercial and specific-pathogen-free (SPF) embryonated chicken eggs used to titer vaccines used routinely in poultry. The aim of this study was to compare the effect of maternal antibodies from the yolk sack of commercial embryonated chicken eggs on the titer of Newcastle disease and infectious bronchitis vaccines.

MATERIALS AND METHODS

Five live Newcastle disease and five live infectious bronchitis vaccines were tittered in duplicate in both, SPF and commercial 10-day old embryonated chicken eggs, according to standard procedures. In brief, all vaccines were diluted 10-fold from 10^{-1} to 10^{-9} (Newcastle disease) or from 10^{-1} to 10^{-6} (infectious bronchitis). Dilutions 10^{-5} to 10^{-9} of Newcastle disease and dilutions 10^{-2} to 10^{-6} of infectious bronchitis vaccines were inoculated into five embryonated eggs via the allantoic fluid, 0.2 mL/egg. After inoculation, all eggs were incubated at 37^{O}C for six days. Allantoic fluid from eggs inoculated with Newcastle disease vaccines were tested for hemagglutination with 2% chicken red blood cells; chicken embryos from eggs inoculated with infectious bronchitis vaccines were tested for typical gross lesions (dwarfing, embryo curled into a spherical form with feet deformed and compressed over the head and with the thickened amnion adhered to it, persistence of the mesonephros containing urates). Vaccine titers were calculated by using the Sperman-Karber methodology.

On day of allantoic fluid or chicken embryo evaluation, yolk sack samples from each egg were taken to perform an ELISA test in order to detect and quantify either Newcastle disease or infectious bronchitis specific antibodies. In brief, yolk sack samples were diluted 1:100 in sterile PBS and then tested by ProFlock NDV plus or ProFlock IBV ELISA test according to the manufacturer's directions (Synbiotics Co., San Diego CA, USA).

Vaccine titers were analyzed by comparison of means; ELISA antibody titers were compared by ANOVA.

RESULTS

Titer of five Newcastle vaccines and five infectious bronchitis vaccines are shown in table 1. There was no statistical difference (P>0.05) between titers of infectious bronchitis vaccines tittered in either commercial embryonated chicken eggs or SPF embryonated chicken eggs. The titer of one Newcastle disease vaccine was higher in SPF embryonated chicken eggs than in commercial embryonated chicken eggs. The mean IBV ELISA antibody titer was 7044 in commercial embryos and 61 (negative) in SPF chicken embryos. The mean NDV ELISA antibody titer was 8305 in commercial embryos and 1507 in SPF chicken embryos. ELISA titers from commercial eggs were higher (P< 0.01) than titers from SPF eggs.

DISCUSSION

Specific-pathogen-free embryonated chicken eggs cost around $1.00 each, while commercial chicken embryos are around $0.30. Results from this study show no difference in titers of infectious bronchitis vaccines when tested in SPF or commercial chicken embryonated eggs, regardless of the high antibody titer found in yolk sack of commercial chicken embryos. The same was seen in Newcastle disease vaccines, except in vaccine C, which titer in SPF embryos was 2 log10 higher than the titer from commercial chicken embryos.

On the other hand, yolk sack samples were suitable for ELISA antibody titration, using a single 1:100 dilution in PBS.

(The whole paper will be published in an indexed journal.)

Table 1. Titer of Newcastle disease or infectious bronchitis vaccines tested in either SPF or commercial chicken embryos, expressed as log10 embryo infecting dose 50/mL.

Vaccine	Newcastle disease		Infectious bronchitis	
	SPF embryo	Commercial embryo	SPF embryo	Commercial embryo
A	6.5	5.9	5.5	5.7
B	6.8	6.4	5.2	4.8
C	9.8*	7.6	5.1	5.3
D	7.6	7.5	5.3	5.7
E	6.3	6.6	5.9	5.8

* $P < 0.05$.

EXAMINING THE DIVERSITY AND ANTIMICROBIAL SUSCEPTIBILITY OF *CLOSTRIDIUM PERFRINGENS* ASSOCIATED WITH ANAEROBIC CELLULITIS IN MARKET-AGE TURKEYS

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SUMMARY

Over the last few years, anaerobic cellulitis has become more prevalent in commercial turkey production. Given the increasing importance of this disease, it is necessary to better understand the microorganisms involved. This study was performed in order to assess the genetic diversity of *Clostridium perfringens* isolated from turkeys experiencing anaerobic cellulitis. Representative isolates were also screened against five antimicrobials commonly used in turkey production to assess their antimicrobial sensitivities. The results of this work suggest a substantial degree of genetic diversity among the isolates tested. Interestingly, isolates from turkeys grown in different states did not cluster independently thereby indicating a dynamic population with many different strains observed in all areas. Penicillin proved to be the most effective antimicrobial tested at inhibiting the growth of *C. perfringens in vitro*, and it continues to be the drug of choice for the control of outbreaks today. The isolates tested here appear to be slightly more resistant to monensin than previously reported.

Anaerobic cellulitis is a complex disease that can lead to excessive mortality in market-age turkeys (2). Similar in its presentation to gangrenous dermatitis in broilers, symptoms of the disease include swelling of the tailhead; accumulation of gelatinous fluid in the abdomen, breast, or inner thighs; severe necrosis of the subcutaneous tissues; and sometimes swelling and accumulation of serosanguinous fluid in the subcutis of the wings (2). The involvement of *Clostridium perfringens* in this disease has been well established (2, 3).

C. perfringens is a spore-forming, Gram-positive anaerobe and is ubiquitous in the poultry production environment. Typically classified into five toxin types based on the production of a combination of four major toxins (α, β, ε and ι), type A strains generally produce the most α-toxin and are the most widespread (8). In addition to α-toxin, *C. perfringens* type A has the ability to produce many minor toxins and enzymes that act synergistically to produce myonecrosis (1).

Many different antimicrobials are currently used in turkey production to promote growth or to treat diseases caused by microorganisms. Three of the more common antimicrobials fed at sub-therapeutic doses for growth promotion are bacitracin, monensin, and virginiamycin. Penicillin and either chlortetracycline or oxytetracycline are often administered to control a cellulitis outbreak. The purpose of this study was to measure the amount of genetic diversity present among isolates of *C. perfringens* associated with cases of

anaerobic cellulitis as well as to assess the sensitivity of *C. perfringens* to commonly used antimicrobials.

MATERIALS AND METHODS

***C. perfringens* isolates.** A total of 196 isolates of *C. perfringens* were selected from Agtech's collection to be examined by RAPD PCR. The isolates were originally cultured from turkeys suspected to have died from clostridial cellulitis. Infected tissues were received from commercial producers in Wisconsin, Minnesota, Missouri, and Virginia. Isolates were re-grown from -84°C freezer stocks in 10 mL Reinforced Clostridial Medium (Difco™) for 24 hrs at 37°C in anaerobic boxes. The isolates were then struck for purity on Perfringens Agar Base (Oxoid LTD) with SR0088E (Oxoid LTD) supplement and 25 mL egg yolk emulsion added per 500 mL of media. After anaerobic incubation at 37°C for 24 hrs an isolated colony was picked into 5 mL pre-reduced Brain Heart Infusion broth (Anaerobe Systems, Morgan Hill, CA) and incubated anaerobically for 24 hrs at 37°C. Cells from 24 hr cultures were harvested by centrifugation at 4500 x g for 5 min.; re-suspended in 1 mL 50 mM Tris HCl, 50 mM EDTA containing 15% sucrose (pH=8.0); and stored at -20°C until DNA isolation.

DNA isolation and PCR. Cells were harvested by centrifugation at 6000 x g for 5 minutes from a 500 µL volume of the cell suspension. The cell pellet was re-suspended in 200 µL 10 mM Tris HCl (pH = 7.5) and DNA was isolated using a High Pure PCR Template Preparation Kit (Roche, Manheim, Germany) according to the manufacturer's protocol except that 100mg/mL lysozyme was used instead of 10 mg/mL. DNA was quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Eugene, OR) and a GENios microplate reader (TECAN, Salzburg, Austria). After DNA isolation each strain was confirmed as *C. perfringens* with a multiplex PCR that targets the major toxin genes (10). Fingerprints were generated by RAPD PCR to assess the genetic diversity among the strains. Each 25 µL reaction contained one RAPD bead, 10 ng genomic DNA and 25 pmol of an arbitrary ten base oligonucleotide primer. Two different fingerprints were created for each isolate using the following primers 5'-GGTGCGGGAA-3' and 5'-GTTTCGCTCC-3'. Amplification for all RAPD analysis was performed in a GeneAmp PCR System Thermal Cycler (Applied Biosystems, Foster City, CA) programmed for one cycle of 4 min at 95°C followed by 45 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. DNA fragments were separated by gel electrophoresis using a 1.0% agarose gel run at 70 volts for 115 minutes. After ethidium bromide staining, gel images were captured using the Syngene BioImaging System and analyzed using Bio-Numerics (Applied Maths Inc., Austin, TX). A composite dendrogram was constructed from the two RAPD DNA banding patterns by using Pearson's coefficient and the un-weighted pair group method with arithmetic averages (UPGMA).

Antibiotic sensitivity screening. Twenty-four representative isolates were chosen for screening against five antibiotics commonly used in turkey production. The five antibiotics tested were virginiamycin (Research Products International Corp., Mt. Prospect, IL), monensin (Sigma Chemical Co., St. Louis, MO), bacitracin (USB Corp., Cleveland, OH), oxytetracycline (Sigma Chemical Co., St. Louis, MO), and penicillin (Sigma Chemical Co., St. Louis, MO). Assays were performed in a 48-well microtiter plate with each well containing 500 µL of pre-reduced brain heart infusion broth (Anaerobe Systems, Morgan Hill, CA). Sensitivity was assessed in duplicate with serial two-fold dilutions of each antibiotic. After anaerobic incubation at 37°C for 8 hrs optical densities at 595 nm were measured using a GENios microplate reader (TECAN, Salzburg, Austria). The minimum inhibitory concentration (MIC) was determined to be the lowest concentration of antibiotic required for >80% inhibition of bacterial growth.

RESULTS

Diversity of *C. perfringens*. All 196 isolates were positive for the α-toxin gene while none of the genes that code for the other three major toxins produced by *C. perfringens* were present. This confirms that all of the isolates examined in this study were *C. perfringens* type A. Thirty-two families were identified at 75% similarity in the composite dendrogram constructed from the results of RAPD PCR performed with the two random primers. Eighteen of these families consisted of three or more isolates and contained 89.3% of the isolates in the dendrogram. The largest family identified included 70 isolates or 35.7% of the total and was the only family to contain isolates from all four states. Five families included isolates from three of the four states sampled. Only seven isolates (>4%) were less than 75% related to any other isolate. Isolates recovered from the same bird were often located in separate families within the dendrogram. Some small families were found in only one state, and were usually isolated from the same bird.

Antimicrobial sensitivity. MIC values that were reproducible are presented in Table 1. Sensitivities to bacitracin and penicillin were the most reproducible. Monensin was the least reproducible of the antimicrobials tested. The MIC values for some strains varied up to eight-fold when tested against monensin. Inconsistent results did not correlate with any particular

strain. Generally, of the five antibiotics examined in this study, bacitracin was the least effective at inhibiting the growth of *C. perfringens in vitro*. Eleven isolates tested required a MIC of 256 µg/mL. However, there were four isolates inhibited in one assay by a 4 µg/mL concentration of bacitracin. Monensin, oxytetracycline, and virginiamycin all showed moderate effectiveness at inhibiting growth, although virginiamycin was very effective against eight isolates tested. Penicillin was consistently the most effective at inhibiting growth. No isolate tested in this study was able to grow in a concentration of penicillin higher than 0.0625 µg/mL. Any isolate not mentioned or included in the table did not produce a consistent enough result to be reported.

DISCUSSION

The results of this work indicate a substantial degree of genetic variability within isolates of *C. perfringens* harvested from tissues affected with anaerobic cellulitis. Thirty-two unique families were identified among the 196 isolates examined. Eighteen of these were comprised of more than three isolates. Although high levels of diversity within this species have been established previously (4, 5, 7), this work shows a substantial degree of genetic diversity associated with anaerobic cellulitis and that multiple strains are involved. It was especially interesting that isolates from different states clustered together but that overall the diversity remained high. One of the mechanisms proposed to explain such genomic variability is through frequent genetic recombination (6). Some virulence factors have been shown to exist in variable genomic locations (6) possibly creating many different genetic fingerprints despite all of the essential genes required for a particular disease process being present. This may explain why the overall population remains diverse with many strains identified in multiple geographic locations. The dynamic nature of the *C. perfringens* genome also suggests an ability to adapt to a multitude of environmental situations.

The prevalence of antimicrobial compounds in poultry production today necessitates periodic monitoring of their effectiveness against field isolates of microorganisms that may develop resistance. The findings presented here are largely in agreement with sensitivities reported elsewhere (9). Penicillin continues to be the drug of choice to control outbreaks of clostridial cellulitis, which is in agreement with the results of this analysis. However, the use of penicillin does present food safety concerns and fails to provide an ideal long term solution. Interestingly, isolates tested in this study showed slightly more resistance to monensin than established elsewhere (9). Although it is possible that *C. perfringens* may be acquiring resistance to this antimicrobial, more work would be required to test this hypothesis. According to this study, virginiamycin and oxytetracycline are moderately effective at inhibiting the growth of *C. perfringens in vitro*. This suggests that oxytetracycline may not be the best option for the treatment of cellulitis unless administered in very high doses. It also suggests that sub-therapeutic administration of virginiamycin may not be enough to reduce *C. perfringens* populations in the production environment. Many of the isolates examined in this study were cultured from turkeys that had received virginiamycin for growth promotion but broke with cellulitis nonetheless. While there remains much to learn about this complex disease and the organisms involved, investigations such as this are a prerequisite for the development of more effective prevention strategies.

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Table 1. Minimum inhibitory concentration (MIC) of five antibiotics for *C. perfringens* strains isolated from turkeys with anaerobic cellulitis (only reproducible values are included).

Antimicrobial	Number of strains with MIC (μg/mL) of												
	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256
Bacitracin	—	—	—	—	—	—	—	—	—	—	—	—	11
Monensin	—	—	—	—	—	2	14	4	—	—	—	—	—
Oxytetracycline	—	—	—	—	1	—	1	5	8	—	—	—	—
Penicillin	7	5	—	—	—	—	—	—	—	—	—	—	—
Virginiamycin	—	8	—	—	—	3	2	—	—	—	—	—	—

THE POTENTIAL WEAPONIZATION OF AVIAN INFLUENZA VIRUS – A POSSIBLE THREAT TO THE COMMERCIAL POULTRY INDUSTRY – DOCUMENTARY EVIDENCE

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OVERVIEW

Avian influenza was the subject of biological weapons research in the former Soviet Union. With its collapse, much of the infrastructure was abandoned or dispersed for sale by organized crime or corrupt officials. Many of the scientists who lost employment were forced to enter the black market. Some were employed by other state based biological weapons programs, while still others are rumored to be working for groups like al Qaeda, Hezbollah and the Al-Aqsa Martyrs Brigades. Current concerns also include the actual biological weapons material. Some of it remains unaccounted for. Although much of the material would likely no longer be viable, there still remains the possibility that some could remain infectious, particularly if given proper storage conditions or re-propagation. Biological weapons programs are thought to be active in at least ten countries, all of which are considered potential adversaries of the United States. Of these programs, most problematic are those in Iran, China, North Korea and Russia. Evidence uncovered during the invasion of Afghanistan and contained in the Department of Defense's Harmony Database clearly indicate that al-Qaeda was discussing the possibility of using avian influenza virus against the United States or her allies.

Evidence uncovered in post-invasion Iraq also indicates that the regime had been working on stabilization of biological materials, although not specifically avian influenza virus (AIV), for delivery via ballistic missiles. Iraq did have an active avian influenza research program, which ironically was documented inadvertently at one point by CNN. Unfortunately, given the problems with rapidly securing the facility, the true nature of the program (offensive or defensive) has not been determined. North Korea and China have recently experienced significant avian influenza outbreaks, lending to the

possibility that infectious materials could be diverted from endemic areas into biological weapons programs. Short of verified human intelligence or direct evidence such as actual materials, "proof" that a biological weapons program is active or focused in a particular direction, will always be extremely difficult to prove, particularly given the scale and nature of some of the modern programs. Current dual use biological technology can always provide a mantle of plausible deniability, since both material and equipment can easily and quickly be diverted from defensive, animal health oriented research to that of an offensive nature designed to kill animals or people. Since currently there is no accurate way of predicting human intentions, intelligence in many forms must be continually gathered, collated and analyzed properly so that a better picture of potential biological weapons programs can be accurately developed.

Giving the sensitivity of the subject, assessing or even discussing potential weapons programs in an open context is very difficult. Much of what has become public is in reality contained within a historical context, lending itself not to the "what is now", but rather to the "what was then". The information contained within the two most famous documents emerging publicly from Afghanistan indicate that al Qaeda operatives did at one time explore the use of animal and plant pathogens, including among others things AIV. Authorship of the "English Documents" has never been disclosed, although at least two specific individuals have been associated with them by intelligence analysts. Beyond these documents, there is actual direct evidence that chemical weapons were experimented with. The public was outraged by images of animal experimentation, which was released by CNN. Actual experimentation of biological weapons, including AIV can only be left to conjecture, since more detailed documents have not been released by the intelligence community. Currently, there is no indication that this actually occurred.

One individual who may have been involved in biological weapons research was a Pakistani microbiologist named Abdur Rauf. Allegedly having al-Qaeda sympathies, this individual is known to have traveled throughout Europe in the latter 1990s, attempting to gather bacterial pathogens. In one cryptic memo he wrote Ayman al-Zawahiri, the number two commander of al-Qaeda, that he had "successfully achieved the targets." In this particular case intelligence experts believe that he was referring to anthrax, since other documents indicate his primary interest was in weaponizing the pathogen. Little else concerning the individual can be discussed in an open context. Nothing indicating a connection with Rauf and avian influenza research has ever been released and is not currently known to exist. It is publicly documented that Rauf holds a degree in microbiology, specializing in food production. Captured and held by the Pakistan government, Rauf has recently been released and currently remains outside the jurisdiction of the United States, although he has been questioned by U.S. authorities.

Members of the Al-Aqsa Martyrs Brigade announced in June of 2006, just prior to the Israeli intervention in to Gaza, that they had developed chemical and biological weapons and were prepared to use them. Claiming in excess of 20 different chemical and biological weapons, the group failed to utilize any of the material in retaliation of the invasion. Many in the intelligence community remain skeptical that such capabilities actually exist within the group, much less a way to effectively deliver the materials. There is no known reference to the group being associated with specific pathogens. There is also no none claim that the group possesses AIV. While to date there has been no verification of any of the claims by Israeli defense officials, the claim is noteworthy because it represents a significant escalation in the rhetoric. It may also indicate that although not currently available, the group may seek such weapons capability in the future.

In summary, there is evidence that some past programs have explored the use of AIV. Most of these programs are no longer active. Other problematic programs remain, several of which originate in areas where AIV would be relatively easy to obtain from naturally occurring cases of the disease. Although, there is no known direct evidence that there is an imminent threat to the United States or her allies, it must remain as a possibility that some of these programs could one day threaten U.S. poultry population. Veterinarians capable of recognizing foreign animal diseases, including avian influenza must be significantly expanded in number. If an attack were to occur from a state based adversary or proxy group, such as al Qaeda, it is unlikely that the attack will be limited in its scope or breadth. The former Soviet strategy of using multiple pathogens simultaneously over large geographical regions should be considered the likely "worst case scenario." Other more limited attacks are likely to be easier to conduct, but far less likely to cause the sufficient effect to warrant the risk to those who intend to carry the attack out.

Terror attacks that do not cause terror cause adversaries to rapidly lose credibility, meaning any attack aimed at a critical infrastructure is likely to be larger, rather smaller in scope. Adversaries, such as al-Qaeda seek more than to just produce a localized tactical victory. Rather, they will seek a strategic victory, meaning the worst case scenario must always be considered a real possibility. Should such an attack

occur in the United States, regardless of the commodity, other commodities or non-agricultural entities will receive collateral damage, if just only economically. Rather than relying solely on federal responses, were a serious and widespread attack to occur, states must be prepared and equipped to deal with the situation largely on their own. Federal agencies will arrive eventually, but initially will most likely be severely taxed keeping the logistical supplies moving, identifying the perpetrators and eventually retaliating militarily in a swift and forceful manner. Remaining eternally vigilant the Intelligence Community must also continue to look for any evidence that biological weapons programs continue and the United States could be targeted. To do so they will need to learn from the expertise that resides in industry and academia. With this partnership of vigilance, there must be a firmer commitment by the government to creating information conduits that flow in both directions. Since the industry remains the target, there is real motivation to prevent disasters before they occur. Besides motivation, industry also has trained eyes in the field and the boots on the ground to more quickly detect anomalies and begin to respond to any emergency, regardless of its source.

AUTOMATIC AVIAN SERUM-SEPARATION DEVICE: MODIFIED PLASTIC STRAWS FOR FIELD COLLECTION

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INTRODUCTION

Blood collection for serum samples is integral in diagnostic testing and regulatory surveillance of disease. Testing for pathogens such as avian influenza, *Salmonella* and *Mycoplasma* is now routine in all regulated livestock industries. Pathogen surveillance is required for interstate shipment, importation of live birds, sales and exhibit. Diagnostic testing can assist in serological profiling, as well as determining the cause of morbidity and mortality experienced in a given flock.

Currently, blood samples are collected into a sterile glass or plastic vials for coagulation. Following coagulation the serum is obtained by subsequently transferring it into a second clean sealed vial. Alternatively, field collection of avian blood samples can be performed in microtest plates or filter papers. However, this method is generally restricted for a few specific serological tests due to the small quantities of serum obtained. Drawbacks for both methods are that these procedures require extra work at the diagnostic laboratory and in the field. In the laboratory, they increase sample handling and in the field sample number must be quite large. Thus, in some instances diagnostic testing can become somewhat time consuming, expensive, and require a large number of vials and shipment boxes.

Herein, an alternative method for *in situ* serum separation using modified plastic drinking straws is described. The use of plastic straws for field collection of blood samples for routine avian serology was first described by McMullin P.F. in 1982 (1). A modified version of these technique was developed by the current author in 1991 (2), and became a wide spread phenomenon in Mexico and others Latin American countries. Yet another improved version has now been developed in order to minimize sample handling and reduce costs, while maximizing the amount of serum obtained. This paper describes the further modification to this technique – The Automatic Avian Serum-Separating Device.

SERUM SEPARATION METHOD AND TECHNOLOGY

The automatic avian serum-separation device. The device consists of a modified plastic drinking straw with three main components: the collection chamber (upper half), the filter (middle), and the serum collection chamber (bottom half). Following blood collection, the device will automatically separate the serum from the clot and upon completion; both the collection chamber and the filter can be discarded. The serum collection chamber can then be sealed, packed, and used for shipping and or storage purposes.

The straws. Conventional plastic drinking straws (25 cm long and about 6 mm in diameter) are sealed by heat at one open end. The remaining open-end is sealed with a small plastic cap. A 1 cm long plastic-rubber filter is placed about 8 cm from the sealed end

making a serum collecting chamber of approximately 2 mL. The upper half is 14 cm holding approximately 3.5 mL of blood sample. As previously described, the straws have been designed so that the filter and the blood collection chamber can be dislodged once the serum is obtained.

The filter. The filter is a special cylindrical plastic-rubber and operates in two ways. Firstly, it holds the blood sample in the upper half while coagulation takes place and secondly, it allows passing of the serum to the bottom collection chamber.

Light portable disposable holder-shipment case. A light and portable-disposable custom-made case has been designed to hold up to ten straws. The case is made of polyethylene terephthalate (PET) and its dimensions are as follows: 26 cm long, 14 cm width and 1.5 cm height weighing around 60 grams. Similar to the straws, once the case is opened it can be transformed into a straw holder for field blood collection and later be transformed in a shipment and storage case.

Blood serum separation proposed technique. The blood sample (up to 3.5 mL) is gently transferred into the opened upper half end of the plastic straw. Once the blood has been transferred the open end is then sealed with the plastic cap provided. Let the sample stand in the straw holder preferably in a vertical position and in a warm place for best blood coagulation results. In general through gravitational forces and vibration movements the serum is automatically filtered. If the serum is not complete filtered, the procedure can be completed by gently squeezing the lower half of the straw (serum chamber below the filter), this creates a vacuum that pulls the remaining serum throughout the filter into the bottom chamber. Once the serum has been filtered, the clot and filter are discharged by cutting the straw and then sealed with the same small cap. The bottom half of the straw (below the filter) now becomes in a "serum vial."

The device presents the following advantages: Separation of the serum *in situ* without the need to transfer the serum obtained into a second tube/vial. Usually the volume of serum obtained is at least 50% of the blood sample. The risk for bacterial growth or bacterial contamination is therefore dramatically reduced. The sealed serum vials with the attached caps ensure the samples do not leak during transit. The PET-case is multipurpose, serving as straw holder, serum storage, shipment case for serum "vials", as well as a holder for laboratory testing. The straw holder facilitates the identification of individual samples in the field as well as the laboratory procedures. The straws and the holder case are light, portable, disposable, and easy to store; consequentially, the shipment and transport cost is reduced.

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ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM AN INTEGRATED POULTRY COMPANY IN MEXICO

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INTRODUCTION

Escherichia coli is a common and important bacterial pathogen that causes at least 5% of the mortality in poultry flocks. Pathogenic serogroups of *E. coli* are ubiquitous in environments in which poultry are raised and can cause airsacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis, cellulitis, or yolk sac infections (YSI). For many years, it was thought that *E. coli* strains associated with YSI cases were avirulent or of low virulence.

Antimicrobial resistance among bacterial pathogens of food animals can complicate veterinary therapy. Resistant animal pathogens may also be a threat to human health if these resistant bacteria enter the food supply or otherwise serve as reservoirs of resistance genes for human pathogens.

MATERIAL AND METHODS

Strains were isolated from an integrated broiler company located at Queretaro State in Mexico. Samples were taken at breeder flock (fertile egg) and hatchery (infertile egg, dead-in-shell embryos, and newly-hatched broilers. Samples from three broiler farms were also collected (2.1, 2.4, and 5.2 weeks). All samples were submitted to the laboratory of the University of Mexico and were seeded onto blood agar and McConkey agar and incubated overnight at 37°C. Lactose positive samples were biochemically tested and strains identified as *Escherichia coli* were stored in Dorset media until further assays were performed.

Strains were recovered from Dorset media, and cultured onto McConkey and blood agar (Difco Laboratories, Detroit Mich.). The obtained cultures were tested again in the VITEK AutoMicrobic System (Vitek AMS; bioMérieux Vitek) to confirm their identity.

Antimicrobial susceptibility of the strains was determined by the standard disk procedure described by Bauer *et al*. The selection of disk concentrations and zone diameter interpretation was done as recommended by the manufacturers and by the National Committee for Clinical Laboratory Standards.

The antimicrobials used in this experiment were enrofloxacin, magnacin, phosphomycin, tiamulin, ceftiofur, doxycycline, phlorphenicol, trimethoprim-sulfachloropiridazine, furazolidone, tetracycline, norfloxacin, trimethoprim-sulfamethoxazole, and ampicillin.

RESULTS

The antimicrobial test revealed a high level of resistance to all evaluated antimicrobial agents. The major resistance frequencies were against enrofloxacin (92.8%), doxycycline (90.7%), tetracycline (90.7%), phosphomycin (89.7%), and phlorphenicol (83.5%). (See Table 1.) On the other hand, the antibiotics that show the lowest levels of resistance were norfloxacin (38.1%), tiamulin (35.1%), ceftiofur (33%), and magnacin (30.9%). All of the strains were resistant to at least one antimicrobial agent.

Fifty-two different multi-resistance patterns were observed among 97 strains tested (86.8%). The most common profile (n=6) was against magnacin, phlorphenicol, phosphomycin, enrofloxacin, tiamulin, norfloxacin, trimethoprim-sulfachloropiridazine, doxycycline, tetracycline, ceftiofur, trimethoprim-sulfamethoxazole, furazolidone, and ampicillin. Six strains showed resistance against all the antimicrobials tested, whereas one strains showed the lowest resistance against two agents (ampicillin and tetracycline).

DISCUSSION

Since 1950, control measures for *E. coli*-associated diseases have depended mostly on the prophylactic and therapeutic use of certain antibiotics. However, this practice has provided a selective pressure for antimicrobial resistance genes; as a result,

many bacteria associated with chickens and poultry meat are now resistant to antimicrobial agents.

The concern of the use of antimicrobials has arisen in the past few years because the total amount of antimicrobials used worldwide is estimated at 100,000–200,000 tons/year, and this exerts a strong selective pressure for the emergence and spread of resistance in both pathogenic and commensal bacteria.

Enrofloxacin is a fluorinated quinolone, which was developed exclusively for use in animals. In the present study, a high rate of resistance to this antimicrobial (92.8%) was observed. Several studies have reported an increase in the resistance to quinolones; however, the percentage in those cases is generally low compared with the percentages observed in this study.

Several studies have been shown that most of the strains isolated from avian colibacillosis are resistant to tetracycline and different sulfonamides. Our results show a high number of resistant strains against quinolones (92.8%) and tetracycline (90.7%). This finding could be related with the different mechanisms for dissemination of genes. The widespread dissemination of antibiotic resistance among bacterial populations could maintain or even increase the number of harmful bacteria involved in infections.

In our study, it was of particular interest the high incidence of multiple-drug resistant strains, since all of the analyzed strains were resistant to at least one agent, and 6% were resistant against all the antimicrobial tested. The recovery of the multiple-drug resistant strains from food animals has been widely documented. In particular, *E. coli* isolated from retail meats has been found resistant to frontline therapeutic antimicrobials such as trimethoprim-sulfamethoxazole, third-generation cephalosporins, and fluoroquinolones.

CONCLUSION

Large plasmids are common among APEC (avian pathogenic *E. coli*) strains and contain genes involved in antimicrobial resistance. Previous studies performed in our laboratory have shown an increase of resistance against the common antimicrobials used in Mexico in the past decade. Nowadays, colibacillosis represents one of the most serious field problems in Mexico because of the high levels of resistance that affect the duration of treatment, mortality, and the number of cull broilers. Thus, the therapy is difficult, and isolates should be tested for antibiotic resistance before treatment since a high proportion of pathogenic isolates of *E. coli* from poultry are resistant to numerous antibiotics.

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Table 1. Percentage of Antimicrobial resistance in different strains isolated from a Mexican poultry integration.

Antimicrobial Agent	Resistant Strains	
	#	%
Enrofloxacin	90	92.8
Tetracycline	88	90.7
Doxycycline	88	90.7
Phosphomycin	87	89.7
Phlorphenicol	81	83.5
Ampicillin	77	79.4
Trimethoprim-sulphachloropiridazine	65	67
Trimethoprim-sulphamethoxazole	62	63.9
Furazolidone	55	56.7
Norfloxacin	37	38.1
Tiamulin	34	35.1
Ceftiofur	32	33
Magnacin	30	30.9

USE OF AN OIL-EMULSION INACTIVATED VACCINE AGAINST H9N2 INFLUENZA VIRUS

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SUMMARY

Since 2000 H9N2 influenza virus was frequently isolated from turkey and chicken flocks in Israel. Single isolates have been recorded in commercial ostrich, geese flock, and in wild pigeon. The actual epidemic began in the Northern part of Israel, mainly in turkeys. Since 2000 the number of infected flocks steadily grew and the virus spread almost all over the country affecting all branches (turkeys, layers, breeders, and broilers). All the isolates were classified as low pathogenic avian influenza viruses (LPAI) by intravenous pathogenicity index (IVPI).

In 2002 it was decided to start using a vaccine against the H9N2 influenza strain. The H9N2 strain was obtained from the Veterinary Services as third passage in SPF eggs in allantoic fluid (AF). This material was passaged twice in SPF eggs and after lyophilization served as a Master Seed (MS). One additional passage in SPF eggs in lyophilized form is used as Working Seed (WS). For production of a vaccine the WS is diluted in sterile PBS, pH 7.2, so that $10^{2.0}$-$10^{2.3}$ EID_{50} per 0.1 mL are inoculated into the allantoic cavity of 10 day old embryonated eggs. In order to receive a reasonable titer, hatching eggs must originate from AI serologically negative flocks. Hatching eggs with HA titer $\log_2 3$ of the yolk and higher can not be used for vaccine production.

The highest titer of the AF is achieved 48-96 hr after inoculation of the eggs, when the embryo mortality exceeds 50%. The AF harvesting is performed after 48 hr and 72 hr from all dead eggs and after 96 hr from the remaining live eggs. There is no correlation between the titer of the AF expressed by EID_{50}/mL and its titer in HA units. The minimum titer required in the inactivated vaccine is $10^{8.0}$ EID_{50}/dose before inactivation. For inactivation the AF is treated with formaldehyde at final concentration 1:1000.

Three weeks after the first vaccination the individual HI titers are not less than $\log_2 4$ and after two vaccinations the individual HI titers are $\log_2 5$ and higher.

In challenge experiments the virus was recovered from the cloacae and oropharynx (80-100%), and not recovered in vaccinated chickens.

IDENTIFICATION OF AVIAN MYCOPLASMAS FROM COMMERCIAL CHICKENS IN MEXICO

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SUMMARY

A survey was conducted in healthy live commercial White Leghorn chickens from 20 to 80 weeks of age in order to isolate and identify different species of mycoplasma in the major egg producing areas in Mexico, Jalisco and Puebla.

Six-hundred samples were obtained from live and healthy commercial chickens directly from trachea with the aid of sterile cotton swabs (300 from Jalisco and 300 from Puebla). Only flocks with more than 200,000 birds each and without a history of live mycoplasma vaccination or antibiotic use were chosen for the survey.

Each sample was diluted in Frey's Medium broth and swabs were discarded. All samples were refrigerated and transported to the laboratory in less

than 24 hr. Then, samples were incubated for 18 days at 37°C. At days 1 and 8, each sample was transferred to solid Frey Medium and incubated for 30 days at 37°C. Plates were observed daily for any growth. Cultures were cloned for three consecutive passes.

Then, isolates were tested for species identification by biochemical tests (glucose fermentation, arginine hydrolysis, and phosphatase activity). According to the results, isolates were divided into three biochemical groups (Table 1).

Glucose positive isolates were tested for the identification of MG or MS by immunofluorescence (IF) (provided by the University of Georgia) and PCR (S16-rRNA).

A total of 226 isolates was obtained from 600 samples. One-hundred thirty-two isolates were obtained from Puebla and 94 isolates from Jalisco. All isolates showed the characteristic "fried egg" colony morphology of mycoplasmas after growth on solid media. The isolation rate was 37.66% or one isolate for each 2.65 samples.

Results of the biochemical tests indicate that Group A (83 isolates) was identified in birds from 40 to 80 weeks of age in both states; Group B (135 isolates) was identified in birds from 20 to 30 weeks of age in both states; and Group C (8 isolates) was identified in birds from 50 to 80 weeks of age in both states. All samples from group A will be tested by urea hydrolysis in search of *Ureaplasma* spp.

Results of the IF tests performed directly from plates indicate that 37 isolates from a total of 83 resulted positive for MG and 71 isolates resulted positive for MS, meaning that even after three passes, some isolates contain the two species. Three isolates were negative for both species.

Results of the PCR performed from broth medium indicate that 42 isolates resulted positive for MG and 72 isolates resulted positive for MS, meaning again that even after three passes, some isolates contain the two species. Three isolates were negative for both species.

In summary, *M. lipofaciens* (group C), *M. gallisepticum*, and *M. synoviae* (group A) were identified from isolates. More studies have to be done to identify three isolates from group A (which can include *M. gallinaceum*, *M. glycophilum*, or *A. laidlawii)* and 135 isolates from group B (which include *M. gallinarum*, *M. iners*, or *Ureaplasma* spp.).

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Table 1. Biochemical groups of *Mycoplasma* spp. obtained from chickens.

Group	Mycoplasma species	Glucose fermentation	Arginine hydrolysis	Phosphatase activity
A	*M. gallisepticum*; *M. synoviae*; *M. gallinaceum*; *M. glycophilum*; *A. laidlawii*	+	-	+/-
B	*M. gallinarum*; *M. iners*; *Ureaplasma* spp.	-	+	-
C	*M. lipofaciens*	+	+	-

ASSESSMENT OF VIRAL COLONIZATION OF MUSCLES FOLLOWING EXPERIMENTAL CHALLENGE WITH HIGH PATH AND LOW PATH AVIAN INFLUENZA VIRUSES IN VACCINATED AND UNVACCINATED TURKEYS

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INTRODUCTION

Avian influenza (AI) represents one of the major concerns for public health that has emerged in recent times. Over the last decade a sharp increase in the number of outbreaks has occurred with a significant economic impact on the poultry sector and the associated repercussions on international trade of poultry products. Control of HPAI has been historically achieved through stamping out policies; however, recent outbreaks occurring in Italy, the Netherlands, Canada, and Asia have shown that the implementation of stamping out and restriction policies alone may result in massive spread and in culling operations affecting millions of animals. Although the control of HPAI through vaccination was not a recommended option, in recent times, due to increased research and successful field experiences, vaccination has recently been included among the tools to be used for controlling AI.

It is now generally accepted that in most poultry species, highly pathogenic avian influenza (HPAI) viruses cause viremia and systemic infection with virus replication in vital organs and in muscle tissue. In contrast, there is little information about the ability of low pathogenicity avian influenza (LPAI) viruses to replicate and persist in meat. The aim of this study was to determine the ability of LPAI and HPAI viruses of the H7 subtype to colonize turkey muscle tissue following experimental infection. In addition the efficacy of vaccination in preventing viremia and thus meat colonization in turkeys was investigated.

MATERIALS AND METHODS

Four groups of 15 (six to seven weeks old) turkeys were used. Two of these groups were immunized subcutaneously (at 19 and 40 days of age) with 0.5 mL of a commercially available inactivated bivalent vaccine (H7N1/H5N9). The other two groups were left unvaccinated. One group of vaccinated birds and one group of unvaccinated birds were then infected intranasally with 106 EID50/100µL of A/turkey/Italy/4580/99 (HPAI). The remaining groups were infected with the same dose of H7N1 A/turkey/Italy/3675/99 (LPAI).

On day 1, 2, 3, 4, and 5 blood (with anticoagulant) was collected from each bird and the presence of viremia was evaluated by real time reverse transcriptase PCR (RRT-PCR). Up to three birds from each group presenting viremia were sacrificed on the day of testing. When blood samples yielded negative results three turkeys were sacrificed randomly. When dead birds were found, organs were collected on the day of death and breast (deep and superficial) and thigh muscle was collected. Samples were analyzed by RRT-PCR and by virus isolation in 9-11 embryonated specific-pathogen-free (SPF) chicken eggs according to EU Directive 92/40 (1).

RESULTS

All unvaccinated HPAI infected turkeys showed severe clinical signs starting from day one post infection with 100% mortality by day four post infection. Virus was detected by RRT-PCR and virus isolation in all of the blood and muscle samples collected from these birds. Vaccinated turkeys challenged with HPAI did not show any clinical signs and none of these birds were found positive for viremia by RRT-PCR. In addition, the muscle samples from these birds yielded negative results both by RRT-PCR and VI.

Unvaccinated turkeys infected with LPAI showed depression and mild conjunctivitis on days three and four post infection. On day two post infection blood samples from two turkeys were positive for viremia by RRT-PCR. This result was confirmed by virus isolation in only one of these birds. No virus was recovered in muscle samples from any of the birds infected with LPAI.

DISCUSSION

Data presented in this paper confirms the ability of HPAI viruses to cause a generalized infection together with colonization of muscle tissue as already reported in other studies (3, 4, 5).

The finding of main interest from this study was the ability of an H7N1 LPAI virus to be detected from the blood. This is in contrast with the general belief that LPAI is a localized infection without viral replication in blood. However, despite the viremia detected, no virus was isolated from the muscle of the infected birds. These results are in partial agreement with a previous report by Kishida *et al*., 2004 which describe the isolation of an H9N2 from imported chicken meat and the detection of virus in blood following experimental infection (3). This suggests that the presence of viable LPAI virus in meat may be strain dependent and that, therefore, further investigations are necessary before any general conclusions are drawn.

The absence of detectable virus in the blood and in meat of vaccinated turkeys infected with HPAI viruses underlines the effectiveness of vaccination in preventing viremia and meat colonization.

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(The full-length article will be published.)

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SALMONELLA SPP. DETERMINATION IN *SALMONELLA*-FREE BREEDER HOUSES BY DIFFERENT SAMPLING METHODS

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ABSTRACT

The necessity of maintaining breeder houses free of *Salmonella* is very important because this way the broiler chickens remain free of *Salmonella* too. This is the reason why continuous sampling must be carried out in order to guarantee the maintenance of *Salmonella*-free birds. The present study was carried out in a breeder open house.

Fifteen drag swabs, fifteen manure samples, fifteen floor eggs, and fifteen broiler chickens of these same breeders were taken. The drag swabs and the manure samples were settled in a pre-enrichment broth and incubated 37°C overnight and later processed according to the Mexican Official Regulation (NOM-005-ZOO-1993). The floor eggs were washed with a phosphate buffered solution for external sampling and then plated on MacConkey and tripticase soy agar. Chicken liver, spleen, gall bladder, duodenum, and egg yolk sac were collected and processed according to the Mexican Official Regulation (NOM-005-ZOO-1993).

There was no isolation of *Salmonella enterica* in this work; nevertheless, we identified other bacterial groups like *Escherichia coli*, *Enterobacter* spp., *Proteus vulgaris*, and *Proteus mirabilis*. This study reinforces the importance of carrying out continuous samplings and biosecurity practices in farms to have *Salmonella*-free birds.

INTRODUCTION

During the last two decades, *Salmonella enteritidis* has caused an increasing pandemia around the world. This pandemia has consequently brought *Salmonella enteritidis* organism to turn into the most common species of *Salmonella* (nontyphoid) in many countries. In fact, *Salmonella enteritidis* causes infection in reproductive system, and the infection lasts during the laying life and the egg becomes infected during the period of its production, even before it is enveloped with a shell. This is why vertical transmission of *S. enteritidis* is very important due to pullet infection.

In integrated poultry organizations, infection of breeder flocks with *S. enteritidis* leads to a rapid dissemination of the organism to progeny broiler and commercial egg laying flocks. *S. enteritidis* is also spread between birds horizontally by the fecal-oral route. The bacterium survives for long periods in the environment and has been isolated from litter and dust in poultry houses. Infection of adult chickens with *S. enteritidis* is largely asymptomatic; however, the organism is readily recovered from the ovaries, oviducts, and ceca of infected birds and from the soft shell and contents of eggs. Also in breeder farms, many sampling methods have been assayed, such as fecal droppings, litter grab, drag swabs, and sock as well as fertile eggs. The necessity to maintain the breeder houses free of *Salmonella* is very important because this way we have broiler chickens free of *Salmonella* too. This is the reason why we must carry out continuous samplings to have the guarantee of maintaining *Salmonella*-free birds.

MATERIAL AND METHODS

Chicken flocks. All fifteen one-day-old liver, spleen, gall bladder, duodenum and egg yolk sac chickens from the breeder flock were macerated separately and inoculated in relation 1:10 in tetrathionate broth as pre-enrichment culture. A loopful from each sample was plated on brilliant green agar to be incubated overnight (37°C).

Drag swabs. Fifteen 8 m x 7.5 m swabbing distance drag swabs from three different places of three breeder houses were taken and placed separately into plastic bags containing lactose broth. Each drag swab was moistened with this broth before taking each sample.

Manure samples. Fifteen manure samples from three different places of three breeder houses were taken and placed separately into plastic bags before being processed.

Drag swabs and manure samples were processed as follows: Each sample was placed in a pre-enrichment broth and incubated at 37°C overnight

and later processed according to the Mexican Official Regulation (NOM-005-ZOO-1993). Manure samples and drag swabs were inoculated (1:10) in tetrathionate broth and a loopful from each sample was plated on brilliant green agar and incubated overnight (37°C). Suspect colonies from each sample were inoculated in biochemical media slants for identification.

Floor eggs. Fifteen floor eggs from different places of three breeder houses were taken and placed separately into plastic bags and, later in the lab, were washed separately with a phosphate buffered solution for external sampling (1:10). A loopful from each egg was plated on MacConkey and trypticase soy agar and incubated overnight (37°C). Suspect colonies from each sample were inoculated in biochemical media slants for its identification.

RESULTS

There was no *Salmonella enterica* isolation from any sample processed in this work; nevertheless, we identified other bacterial groups like *Escherichia coli* and *Proteus mirabilis* from manure samples; *Enterobacter* spp., *Proteus vulgaris*, and *Proteus mirabilis* from drag swabs; *Escherichia coli* from floor eggs; and *Escherichia coli*, *Citrobacter freundii*, and *Proteus mirabilis* from one-day chicken organ samples.

DISCUSSION

Several studies have compared the sensitivity and power of sampling methods. This may be due to changes in *Salmonella* excretion in birds. Most studies which describe how different factors may influence the *Salmonella* excretion rates in chickens are experimental.

Some researchers have described that litter sampling methods that incorporate stepping on the sample material while in contact with the litter appear to detect *Salmonella* in greater incidence than traditional sampling methods of dragging swabs over the litter surface. In this work 8 x 7.5 m swabbing distance drag swabs instead of 3 m or more long distance repetitions were taken, and no *Salmonella* was found in manure samples, floor eggs, or chickens. Nevertheless, in relation to other bacterial groups identified in this work, *Proteus* spp are found in many breeder samples when birds are beginning their production or are at peak lay, maybe because of the stress. Many times *Proteus* spp are isolated from this kind of situation and may compromise bird health.

This study reinforces the importance of carrying out continuous sampling and biosecurity practices in farms in order to have *Salmonella*-free birds. High standards of management and flock biosecurity throughout the life of the flock are also necessary to prevent transmission of *Salmonellae*. These measures include good personal hygiene and sanitary practices, thorough cleaning and disinfection of poultry houses and equipment between flocks, effective manure and sewage disposal, effective control of rodents and other pests, exclusion of poultry by-products from feedstuffs, use of properly processed feedstuffs, and use of eggs/chicks from monitored breeding flocks.

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APOPTOSIS AND ALTERATION IN PARENCHYMA/STROMA RATIO IN LYMPHOID TISSUES OF CHICKEN INOCULATED WITH THE INFECTIOUS BURSAL DISEASE VIRUS

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SUMMARY

Apoptosis is an active and individual cellular death characterized by nuclear and individual cytoplasmic fragmentation into apoptotic bodies (3). In contrast of the necrosis, there is no inflammation in neighboring of the apoptotic cells because there are no release of the cellular contents into the interstitium and, consequently, no activation of inflammatory mediators (11). Little has been published on apoptosis in the lymphoid system of birds. However, it is well accepted that the involution of thymus and bursa of Fabricius are mediated by apoptosis.

The infectious bursal disease (IBD) is an acute and highly contagious viral infection of young birds, which affect particularly the B-lymphocytes of the bursa of Fabricius (BF). Subclinical infection with the IBDV is still a problem to poultry industry and its diagnosis must be supported by histopathology of the bursa of Fabricius (BF), determination of the level of antibodies or by isolation and viral characterization. Infectious bursal disease virus (IBDV) causes intense immunosuppression (6, 7). The immunosuppressive effect of the IBDV is related to the morbidity and mortality of birds (8) and susceptibility to other infections (8). Such immunosuppressive effect causes great economic losses for the poultry industry. Immunosuppression, at least in part, is due to increased apoptosis in BF (9, 10). However, the direct relation of apoptosis and the bursal and lymphoid atrophy in early infections is still unclear.

To address that issue, thirty one-day-old SPF chicks were used in an experiment; twenty-five of them were inoculated with IBDV. Birds were distributed in five experimental groups of six animals each: Group 1: control - non infected birds euthanized at the beginning of the experiment; Groups 2 to 5: infected birds euthanized at different post inoculation periods: 24 hours/Group 2, ; 48 hours /Group 3; 72 hours /Group 4; and 96 hours /Group 5. Before euthanasia, birds were weighed and evaluated. After decapitation they were submitted to autopsy. Fragments of the BF and other lymphoid tissues were harvested for histological processing. Sections 5μm-thick were stained with hematoxylin and eosin (HE) and Gomori´s trichrome for histomorphometrical evaluation of apoptosis, fibrosis, atrophy, and alterations in alteration in parenchyma/stroma ratio in lymphoid tissues. Other fragments of the BFs were stored in microtubes of 1.5 mL at –80ºC, for posterior DNA extraction and electrophoresis in agarose.

The identification and quantification of apoptosis was accomplished by light microscopy. Cellular shrinkage, condensed chromatin in perinuclear clumps and the presence of apoptotic bodies were considered as morphological evidences of apoptosis (5). Morphometry (parenchyma/stroma ratio and apoptotic index) was achieved with a light microscope equipped with a camera connected to a computer (Image analyzer). Data were collected with the aid of an image analyzer (Kontron KS-300 v2.0, Zeiss Kontron Electronics GMBH). Data were analyzed statistically by the non-parametric Kruskal-Wallis test (parenchyma/stroma ratio and apoptotic index). The parenchyma/stroma ratio is a great parameter to evaluate atrophic states. It was determined with low magnification (4X objective). Three fields for slide were used. In each field, follicular area and empty space were measured. Stroma area was obtained by subtracting these areas (follicular area and empty space) from the total area of the field. Slides stained with HE were used to determine the apoptotic index (number of apoptotic cells / total number of cells).

Some slides were also used for *in situ* identification of the fragmentation of the DNA by inserting labeled nucleotides in the 5'OH terminal portion of fragmented DNA of the cells in apoptosis (TUNEL or Terminal deoxynucleotidil transpherase Uracil Nick End Labeling). Apoptosis was confirmed in apoptotic lymphocytes scattered in bursal follicles of the infected groups. Positive labeling was seen as brownish to dark intranuclear dots, as revealed by the diaminobenzidine (DAB) by peroxidase reaction.

Also electrophoresis of bursal DNA in agarose gel showed the internucleosomal DNA fragmentation characteristic of apoptosis. The gels showed the typical

"ladder pattern", by the presence of bands with 200 bp or their multiples. Awhile the infected groups presented more numerous and more intense distal bands, the control group showed denser bands near the migration origin, characterizing lower internucleosomal degradation.

The apoptotic indices were higher in infected groups ($p< 0.001$) that in the control, and showed a progressive increase, at the different post inoculation periods (24, 48, 72 and 96 hours) except between 24 and 48 hours. Therefore IBDV induces hypotrophy of the bursa of Fabricius by the progressive activation of apoptosis. Bursal lymphoid follicles of Control Group were all well cellularized, and both cortical and medullar areas were more defined and denser than in infect groups. The parenchyma/stroma ratio in the BF showed increased stroma in groups 3, 4, and 5 when compared with the control group and also in groups 3, 4, and 5 when compared with group 2. Differently from controls, increased goblet cells were observed in the coating epithelium in infected birds. Also a diffuse and moderate depletion of lymphocytes in the medullar and cortical area were observed, originating countless empty spaces. Apoptotic bodies were frequently found within the empty spaces as well as lymphocytes with condensed chromatin. Arai *et al*. (2) reported induction of apoptosis by radiation in bursal lymphocytes, with morphology of apoptotic cells very similar to the ones we saw in this experiment.

Summarizing, there are evidences that the IBDV causes fragmentation of the DNA and increases the apoptotic index in BF very early after infection. These results allowed us to conclude that apoptosis may be involved in the mechanism of lymphoid depletion caused by IBDV in broiler chicks. Apoptosis is an early event in IBDV infection, present at the very first moments after viral inoculation, indicating that it is more than just a mechanism to explain immunosuppression and bursal atrophy in late IBD. Also it was demonstrated that as long as apoptosis increases, viral protein expression decreases, which suggests that apoptosis plays a role as a defense mechanism against viral replication. The increase of apoptosis may be interpreted as a potential mechanism of protection against the viral response, since the virus needs active and live cells to replicate itself and to progress in infection.

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PREVALENCE OF LOW PATHOGENIC AVIAN INFLUENZA IN SOUTHERN CALIFORNIA LIVE BIRD MARKETS

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SUMMARY

Oral/pharyngeal and cloacal swabs were collected from birds in three live bird markets (LBM) located in Los Angeles County to estimate the prevalence of low pathogenic avian influenza (AI) after detection by routine surveillance in the fall and winter of 2005 using a stratified systematic sampling method. LBMs can act as a center for co-mingling of birds from many different sources, spreading diseases easily between birds. Surveillance activities within the LBM system are important for early detection; however, little is known about the efficacy of the currently employed AI surveillance methods.

RT-PCR was performed on a 320, 65, and 60 samples taken from each market in which number of samples collected depended on the number of birds, age of the birds, and number of species present at the time. Further H and N-typing was performed on a smaller set of samples that were positive by RT-PCR. The sampling methods used to estimate prevalence in this study shows that the surveillance methods currently used within the live bird market system in Southern California is effective to detect disease when prevalence is as low as 0.5% in a LBM with Japanese quail or 3.1% in a LBM with multi-age and breed chickens.

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COMPARISON OF THE HEMAGGLUTINATION INHIBITION TEST AND THE ENZYME LINKED IMMUNOSORBENT ASSAY FOR ANTIBODY TO NEWCASTLE DISEASE IN NON-COMMERCIAL LAYERS RAISED IN BACKYARD FLOCKS OF POOR FAMILIES AND A COMMERCIAL FARM FROM ARGENTINA

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SUMMARY

A commercial Newcastle disease virus (NDV) enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition test (HI) were performed to serum samples for the detection of NDV antibodies. The layers belong to a social program called *Pro-Huerta*, in which chickens are given to poor families. At the same time, sera from a commercial layer farm were obtained. Since Argentina has been declared free of velogenic NDV with vaccination since 1997 and backyard birds can be a potential source of the virus, this study was not only to check for the presence of

antibodies, but also to compare the results obtained between the ELISA and the HI tests. Due to the economic situation of the country, the possibility of running an ELISA as was done in previous years is somehow complicated, and the HI could be another way of helping sanitary controls.

INTRODUCTION

Newcastle disease was diagnosed for the first time in Argentina in 1961. The last outbreak of the disease produced by a velogenic strain was reported in the province of Entre Rios in 1987 and since 1996 only lentogenic strains are being used as vaccines.

There is a social program for food security called Pro-Huerta that belongs to INTA (Instituto Nacional de Tecnología Agropecuaria) where chickens are given to poor families. The present study was done not only to evaluate the sanitary status to Newcastle disease virus (NDV) and recommend ways of control, but to compare the ELISA and HI tests which were performed for the detection of NDV antibodies. The program provides ten chickens per family so that they can raise them in their backyard to produce food for themselves. However, these birds can be a potential source of NDV since Argentina has been declared free of velogenic or exotic NDV with vaccination in 1997.

The very low income families with Pro-Huerta´s chickens were visited and serum samples were obtained. Parallel samples were also obtained from a commercial layer farm. Due to the economic situation of the country, the possibility of running an ELISA as was done in previous years is somehow complicated, and the HI could be another way of helping sanitary controls.

MATERIALS AND METHODS

A commercial NDV ELISA was performed on 400 serum samples to check for NDV antibodies. Families with chickens that received the benefit of Pro-Huerta were visited and serum samples were obtained from those chickens. Additionally, 60 samples were obtained from layers of a commercial farm with a known vaccination program. These layers received three NDV vaccines, but were of different ages: the first 20 were 87 weeks of age, the following 20 were 36 week-old, and the last 20 were 43 weeks of age.

The presence or absence of antibodies to NDV by ELISA is determined by reading the A (650) value of the unknown to the positive control mean. The relative level of antibody in the unknown is determined by calculating the sample to positive ratio (S/P). Serum samples with S/P ratios of less than or equal to 0.2 should be considered negative (FlockCheck, IDEXX). HI tests were also run on the sera.

RESULTS AND DISCUSSION

All commercial layers showed a titer greater than 0.2 due to vaccination. Nevertheless, the older the birds, the lower the titers because they only received three NDV vaccines. A few of the backyard hens should be considered positive and exposed to NDV since they were not vaccinated and no evidence of disease was observed. Good correlations were determined between ELISA and HI tests.

Previous results obtained from hens that do not belong to this program were presented during the last AVMA meeting (1) and part of the results presented here was shown at the WPDC in 2003. Although it is very difficult to accomplish a vaccination program in backyard chickens, a booklet was written by students who collaborated during the sampling to be given to those families together with personalized instructions (2). However, since the Pro-Huerta chickens are not the only backyard birds raised, it would be important to continue to periodically control backyard poultry, and the HI proved again to be a good alternative method.

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